GENETIC ANALYSIS OF INHERITED RETINAL DYSTROPHIES

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DECLARATION

I, Michelle Erica McClements, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

ABSTRACT

This thesis describes the genetic analysis conducted to investigate the cause of six autosomal dominant macular dystrophies (North Carolina macular dystrophy, MCDR1; North Carolina-like macular dystrophy, MCDR3; North Carolina-like macular dystrophy with progressive sensorineural hearing loss, MCDR4; progressive bifocal chorioretinal atrophy, PBCRA; bull's-eye maculopathy, MCDR2 and split-hand/foot malformations with associated North Carolina macular dystrophy, SHFM and NCMD) and one cone dysfunction with associated myopia and dichromacy (Bornholm eye disease, BED). The method of using Affymetrix SNP chips was tested for its usefulness in conducting genetic analysis on the macular disorders. The chips were used to see if disorders previously linked to large genomic regions could have their loci refined to help determine where the genetic error for each disorder lies. The genotyping results reveal the analysis is more informative when including genotyping data of affected offspring and their parents. MCDR1 analysis without such samples did not refine the disease locus. The copy number variation (CNV) analysis conducted was novel for the disorders and highlighted interesting regions of CNV, particularly in the SHFM and NCMD analysis at 5p15.33 for which QPCR confirmed loss of one copy of a novel microRNA. For MCDR2 analysis, new families were identified as carrying the mutation Arg373Cys in exon 10 of the *PROM1* gene. Genetic analysis conducted on various families with BED has revealed the genetic cause of the disorder. Genetic changes leading to the amino acid combination of leucine, isoleucine/valine, alanine, valine and alanine at residues 153, 170, 174, 178 and 180, respectively, in the L or M opsin genes were consistently found in BED patients and are believed to result

in the disease phenotype. Additionally, a family with a BED-related phenotype was found to carry a novel mutation in exon 2 of a hybrid M opsin gene that would lead to a Glu41Lys substitution.

ACKNOWLEDGEMENTS

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1. THE RETINA

Structure and Function

1.1 The Eye

The eye is a complex organ composed of many sections and cell types, all of which need to work together to enable good vision. It is an area of the human body where destruction of only a few cubic millimetres of tissue can have a dramatic impact upon the function of the organ and in turn, the lifestyle of an individual. The eye can be divided into three main concentric layers: the outermost comprises the cornea and sclera; the middle section contains the choroid, ciliary body and iris with the innermost layer being the retina (Figure 1.1).

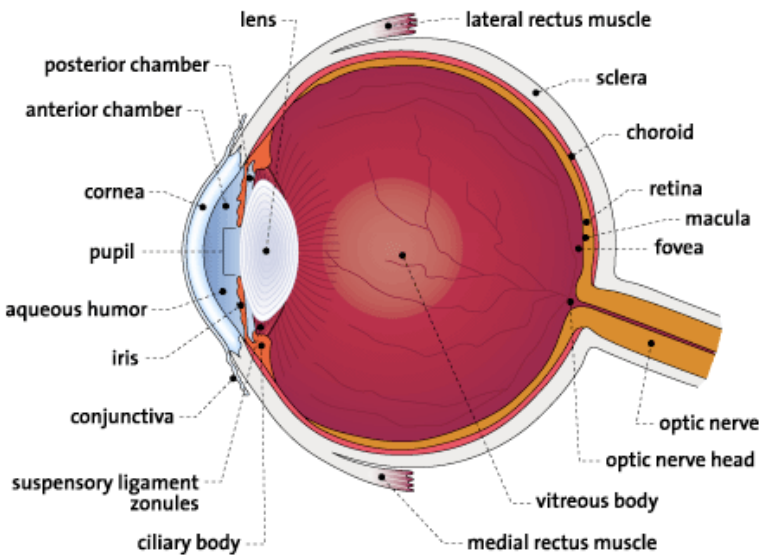


Figure 1.1 A schematic overview of the basic eye structures. Image taken from: www.allaboutvision.com.

1.1.1 The Inner Layer

The retina is a thin multi-layered structure upon which light must be accurately focussed in order to produce a good visual image. As seen in Figure 1.2, the retina is composed of three basic layers of neural tissue and light must pass through the inner and middle layers before it reaches the photoreceptors (rods and cones, the light-sensitive cells that convert light images into electrochemical signals) in the outer layer. The inner layer of the retina is often referred to as the ganglion cell layer (GCL), which is followed by the inner nuclear layer (INL), an area which also contains blood vessels that form the inner retinal vasculature. The outer nuclear layer (ONL) ends with the photoreceptor outer segments, beneath which is the retinal pigment epithelium (RPE) followed by Bruch's membrane, the choroid and the sclera.

1.2 The Retina

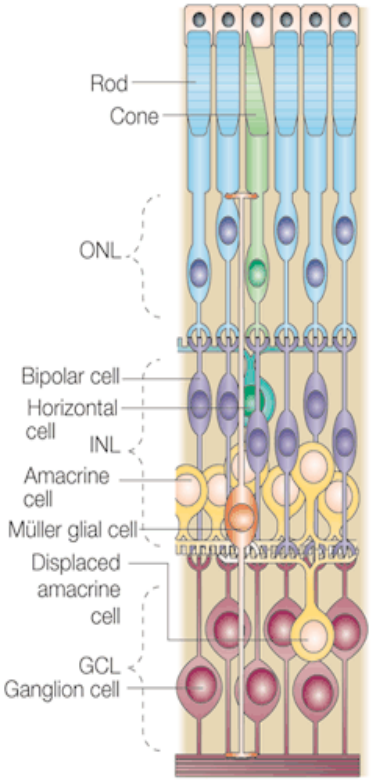


Figure 1.2 General structure of the vertebrate retina taken from Dyer and Cepko (2001). ONL = outer nuclear layer made up of photoreceptor cell bodies; INL = inner nuclear layer, containing bipolar and Müller glial cell bodies; and GCL = ganglion cell layer, which also contains displaced amacrine cell bodies.

The role of the retina is to convert images from the eye's optical system into electrical impulses that are sent along the optic nerve to the brain to interpret as vision. The light sensitive neural tissue of the retina is composed of many cell types with the inner layer of the retina (ganglion cell layer, GCL) consisting of neural connections between bipolar cell axons and ganglion cell dendrites. Some retinal ganglion cells (RGCs) are intrinsically photosensitive (ipRGCs) and use melanopsin as a light sensitive pigment to transmit monosynaptic projections through the retinohypothalamic tract directly to the suprachiasmatic nucleus in the brain. These ipRGCs constitute less than 2% of the total RGC population yet light information for synchronisation of circadian rhythms and pupillary responses are predominantly conveyed through these cells (Güler et al., 2008). The ipRGCs also receive synaptic input from the photoreceptor networks, thus allowing the photoreceptors access to the brain circuits mediating non-image visual functions, circadian rhythms and pupillary responses (Wong et al., 2007).

Following the GCL is a region containing bipolar, Müller, horizontal and amacrine cell bodies, known as the inner nuclear layer (INL). Müller cells are radial glia of the neural retina whose processes span the three cellular layers of the retina by forming an anatomical and functional link between the retinal neurons and the blood vessels, vitreous body and sub-retinal space (Bringmann et al., 2006). Müller cells are important for the maintenance of retinal structure and function and for cone retinal cycling (Section 1.2.5). Selective Müller cell destruction causes retinal dysplasia, photoreceptor apoptosis and retinal degeneration and proliferation of the RPE (Dubois-Dauphin et al., 2000). Horizontal and amacrine cells make lateral connections and modulate the direct signalling pathway from photoreceptors

to ganglion cells. They lack any extension resembling an axon and have only dendrites, though a number of these are pre-synaptic and thus act similarly to axons. The amacrine and bipolar cell dendrites form synapses with photoreceptor axons and the photosensitive columns of rods and cones then form the outer segment of the retina.

1.2.1 Visual Pathways

The combination of cells signalling from each retinal layer to the next form different visual pathways. The *vertical pathway* is formed when a single photoreceptor cell forms a synapse with a bipolar cell (a vertical neuron). The dendritic end of the bipolar cell receives input from the photoreceptor and the axon terminal is then pre-synaptic to a ganglion cell. The dendrites of a single ganglion cell integrate several bipolar inputs and generate an action potential. However, cone photoreceptors often contact several bipolar cells, so in this situation a signal from one cone cell goes down several vertical pathways and is known as a *divergent pathway*. Outside the fovea it is common for a number of photoreceptor cells to connect to a smaller number of bipolar cells which are then pre-synaptic to a single ganglion cell. This is known as a *convergent pathway* and is useful for producing a visual image in low light conditions. There are also *lateral pathways*, which are formed in the inner retina by amacrine cells connecting vertical pathways thereby allowing bipolar and ganglion cells to interact, and in the outer retina by horizontal cells making connections between photoreceptors. The light information from each visual pathway leaves the retina via the axons of ganglion cells that collectively form the optic nerve.

1.2.2 Development of the Retina

In humans, eye formation begins at around 22 days of development and is not completed until several months after birth (Mann, 1964). During embryogenesis the retina (and the RPE) is derived from neural ectoderm whereas the cornea and sclera develop from mesoderm and surface ectoderm produces the lens.

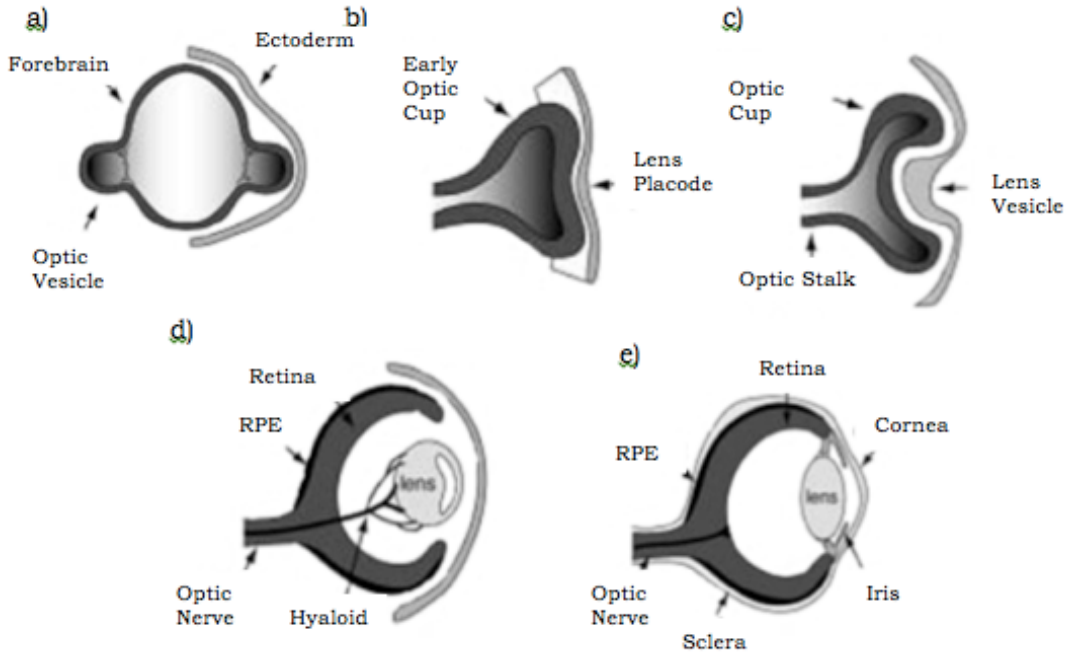


Figure 1.3 Images taken from Wong et al. (2006) showing the basic stages of eye development: a) formation of optic vesicles; b) interaction of the surface ectoderm with the optic vesicles producing the lens placode; c) formation of the optic cup and lens vesicle; d) the RPE and retina are formed and; e) the mature eye.

The retina and optic nerve originate as paired optic vesicles formed as out-pockets on either side of the forebrain (Figure 1.3a), and thus the retina forms part of the central nervous system. Inductive processes between the surface ectoderm and optic vesicles causes the epithelium to thicken and form a lens placode

(Figure 1.3b), the precursor to the lens, which requires *SOX2* and *POU2F1* gene expression (Donner et al., 2007). *SOX2* encodes an SRY-like (sex-determining region Y) HMG (high-mobility group) box transcription factor that mediates its effects by forming complexes with specific co-factors and is critical in vertebrate development. In humans, a *SOX2* mutation has been shown to cause anopthalmia, hearing loss and brain anomalies (Hagstrom et al., 2005). The study by Donner et al. (2007) identified Oct-1 (encoded by *POU2F1*) as a co-factor of Sox2 in mouse lens and nasal placode induction. The genetic combination of Sox2 and Pou2f1 mutant alleles results in ocular and nasal phenotypes that resemble those of the Pax6 null embryos. *PAX6* (paired box gene 6) is one of the most important genes in vertebrate eye development, it is a homeobox gene expressed in the eye field at the early neurula stage of development and proposed to be at the top end of the genetic cascade governing eye development (Lupo et al., 2000). Mutations in *ey*/*pax6* (fly homologue, *ey* = *eyeless*) causes malformation or lack of eyes in *Drosophila*, mouse and humans (Ton et al., 1991; Quiring et al., 1994). According to Barishak and Ofri (2007), the *PAX6* gene product is expressed toward the end of gastrulation in the anterior neural plate and then through the stages of the optic vesicle (though it is not required for optic vesicle formation), optic cup and later on in the retina (in ganglion and amacrine cells). Donner et al. (2007) demonstrated that Pou2f1, Sox2 and Pax6 appear to be interdependent components of a molecular pathway utilised in both lens and nasal placode induction in mice. Formation of placodes is common in the development of vertebrate sensory structures including the lens, ear and nose, which develop through multi-step inductive processes involving formation of a neural plate stage pre-placodal region and subsequent formation of discrete ectodermal thickenings that are the placodes

(Brugmann and Moody, 2005). Links between the formation of the sensory structures may be important as demonstrated by one of the disorders studied here where an early-onset macular dystrophy is associated with progressive sensorineural hearing loss; genes involved in both eye and ear development will be strong candidates in this investigation.

When the lens placode has begun to form, the optic vesicle expands and begins to invaginate to form the optic cup (Figure 1.3c) and as it does so it creates a fold along its centre known as the choroid fissure (Sadler, 1990). This encloses a small amount of angiogenic mesenchyme that later forms the hyaloid artery and vein which develop into the main artery and vein of the retina (Figure 1.3d). As the cup invaginates and folds, two layers arise: the inner layer that will become the retinal tunic (including the light-sensitive elements), and the outer layer that becomes the RPE. Differentiation of retinal cells begins at the primitive zone in the centre of the cup of neuroectoderm and gradually extends toward the rim. This results in retinal ganglion cells and amacrine cells being generated first, followed shortly after by horizontal cells that differentiate in the outer retina whilst the photoreceptors are formed in overlapping phases (Choy et al., 2002). The vertical inner and outer networks are later interconnected when bipolar and Müller cells are formed in the last part of retinogenesis and connections with ganglion cells are established (Young, 1985). As the retina continues to develop there is lateral displacement of the inner retinal neurons and glia, which occurs during late foetal to early infant stages, to create the foveal depression (Hendrickson and Youdelis, 1984). This is the central point on the retina where light from the central visual field strikes and is the region with the greatest visual acuity due to the fact it contains tightly-packed cone photoreceptors. Cones provide us with colour vision but are

only active in relatively bright light whereas rods enable monochromatic vision in low light conditions. The distribution of rods and cones within the retina is well known, with cone density being greatest at the fovea and decreasing rapidly at any distance away from this point. Rods, however, are absent at the fovea but are in abundance nearby and their density gradually decreases with distance from the fovea. For the high visual acuity found in the central retina there is a low ratio of photoreceptors to ganglion cells.

1.2.3 Photoreceptors

The transduction of light into nerve signals sent via the optic nerve to the brain takes place in the photoreceptors and both the cones and rods that undertake this role consist of the same three main parts: an outer segment and inner segment that reflects their ciliary origin (Lamb et al., 2007), and a synaptic terminal. The outer segment is formed of discs containing photopigments, the inner segment contains the nucleus and majority of the organelles within the main cell body whilst the synaptic terminal contains the synaptic vesicles that store the chemical transmitters used in neural communication. The shape of the outer segments gave rise to the naming of the two photoreceptor types; rods have a long, cylindrical outer segment with many discs whereas cones have, by comparison, a short stack of discs that tapers distally (Figure 1.4). Due to the greater number of discs containing photopigment, rods are over a thousand times more light-sensitive than cones and can detect even a single photon of light. The connections rods make with the other cells of the retina also contribute to their sensitivity (Section 1.2.1) but the result of these connections is that the vision provided by the rods is more blurred than that from cones.

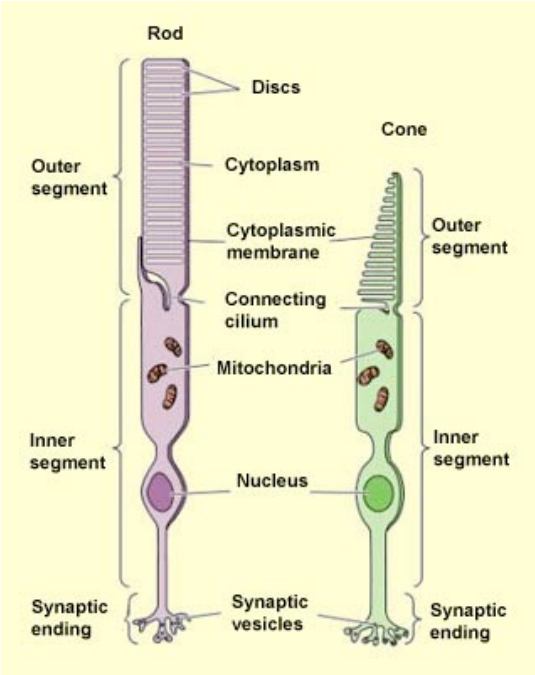


Figure 1.4 A schematic of a rod and cone photoreceptor cell, taken from: http://thebrain.mcgill.ca.

The discs in the outer segments are formed from the folding of the photoreceptor membranes. In rods, the discs become completely detached from and float within the membrane but in cones the discs remain attached to the outer segment membrane. New discs are constantly being formed at the base of the outer segments and the old discs at the tip of the outer segment are recycled. The RPE is responsible for the phagocytosis of the old outer segment tips in addition to numerous other roles considered in Section 1.3.

Whilst physiologically distinguishable, the two photoreceptor cell types are also functionally distinguishable due to their photopigments. The photopigment molecule is formed from a light-absorbing portion, or chromophore, and a protein called an opsin. Rods contain only the A1 chromophore that when bound to the opsin is commonly referred to as rhodopsin, whereas the outer segments of cones contain one of three different iodopsins (rhodopsin analogs) that best absorb light at a particular range of wavelengths; short (S, λ_{max}420nm), medium (M, λ_{max} 535nm) and

long (L, λ_{max} 560nm). S-wavelength selective opsin is found in 8-12% of cones except in the fovea, where they are absent (Curcio et al., 1991). These cones are blue sensitive, M cones green sensitive and L cones red sensitive and the photopigment in rods is for visual purple (λ_{max} 498nm) used in scotopic vision (vision in dark conditions). The cone photopigments (chromophore plus opsin) have different absorption spectra despite containing the same chromophore. The differences in the spectral characteristics are dictated by the interactions of specific amino acid residues of the opsin with the chromophore, a process known as spectral tuning (Section 1.2.4). Whilst the general distribution of rods and cones is known, what is less well determined is the layout of the three cone types, known as the trichromatic cone mosaic.

The S cone sub-mosaic has been well characterised (Curcio et al., 1991) but the organisation of L and M cones less so because they have no known histochemical differences and their pigments are 96% identical (Nathans et al., 1986b). Bowmaker et al. (2003) reported that L and M cones were found in random patches in the human retina, yet there is also evidence suggesting non-random arrangements as both Hagstrom et al. (1998) and Bowmaker et al. (2003) found the L to M ratio increases in the far periphery of the retina. Combining the techniques of high-resolution adaptive optics imaging and retinal densitometry, Hofer et al. (2005) characterised the S, L and M cone arrangements in eight human foveal mosaics. They determined that the normal male L to M ratio varies (1.1:1 to 16.5:1) whilst all subjects had nearly identical S densities. The L and M cones appear to cluster together but otherwise do not seem to have a regular pattern.

In foetal retina there are more S cones than there are in adult retina and a significant proportion of cones in foetal retina have

been shown to express S and L or M opsin (Cornish et al., 2004). The L/M cones appear in the foveal cone mosaic 3 to 4 weeks after S cones and by birth are present throughout the retina. There is little evidence of significant levels of apoptosis in the L/M cones so it is thought that cones destined to express L or M may initially and transiently express S before switching to L or M (Cornish et al., 2004).

1.2.4 L and M Photopigment Genes

The S opsin gene is located at 7q32.1, whilst the L and M genes are found in a tandem array downstream of a locus control region (LCR) at Xq28 (Vollrath et al., 1988). The array consists of one L gene followed by one or more downstream M genes (Nathans et al., 1986b; Macke and Nathans, 1997). Both L and M alleles are present in each cone but in the adult retina only one allele is expressed (Hagstrom et al., 2000). It is widely believed that expression of the array includes only the first two genes in the cluster (Winderickx et al., 1992a). The identification of the LCR by Nathans et al. (1989) gave insight into why this may be the case; it is thought that the proximity of the genes to the LCR influences expression. Hayashi et al. (1999) showed that if the transcription start sites were ~3.5kb and ~43kb from the LCR they were efficiently activated but at ~82kb away they were not. The LCR is upstream of the array and required for expression of both L and M genes though each gene in the array has its own promoter. The head-to-tail array seems to have arisen from a gene duplication (Dulai et al., 1999) and the LCR is an enhancer that lies between 3.1kb and 3.7kb upstream of the translational start site of the first gene. Deleting the LCR prevents expression of all the genes in the array and results in blue cone monochromacy (Nathans et al., 1989). The LCR is proposed to act as a stochastic selector that chooses which photopigment gene will be expressed in an

individual photoreceptor (Smallwood et al. 2002). The model proposed by Smallwood et al. (2002) suggests that the L and M promoters compete for contact with the LCR and the promoter of the randomly chosen gene forms a stable and permanent complex with the LCR. This model may be contested though because the LCR is present in New World monkeys where only a single gene is present (Dulai et al., 1999).

Hennig et al. (2007) reviewed the regulation of photoreceptor expression in mice and described how it is mediated by a network of photoreceptor transcription factors centred on cone-rod homeobox protein (Crx), an Otx-like homeodomain transcription factor. The network is cell type-specific and governed by factors preferentially expressed by rods (for example: neural retina-specific leucine zipper protein, Nrl and; nuclear receptor subfamily 2, group E, member 3, Nr2e3) or cones (for example: thyroid hormone receptor β2, Trβ2; retinoid related orphan receptor, Rorβ and; retinoid X receptor, Rxrγ). The process also depends on general transcription factors and co-factors. Mutations that interfere with any of the interactions can cause photoreceptor development defects or degeneration (Section 2). Selective expression of the cone opsin genes is important for development of photoreceptors and for maintaining integrity and function. Crx regulates transcription of many photoreceptor-specific genes (Furukawa et al., 1997). Nr2e3 interacts with Crx and has opposing effects on transcription of rod and cone genes. HEK293 cells with transient expression of both demonstrated that Nr2e3 enhances rhodopsin but represses S or M photopigment transcription when interacting with Crx. Mutations in human NR2E3 cause enhanced S-cone syndrome (ESCS, Haider et al., 2000).

Crx has been shown to interact with co-activators with histone acetyltransferase (HAT) activity. Peng and Chen (2007) suggest the transcription sequence of events in promoter and enhancer regions is as follows: 1. binding of Crx; 2. binding of HATs; 3. acetylation of histone H3 (AcH3) and 4. binding of other photoreceptor transcription factors (eg Nrl and Nr2e3) and RNA polymerase II. In Crx knockout mice, association of HATs and AcH3 with the target promoter/enhancer regions is significantly decreased, correlating to aberrant opsin transcription and photoreceptor dysfunction. Crx mutations are known to cause cone-rod dystrophy (CORD), leber congenital amaurosis (LCA) and retinitis pigmentosa (RP) (Freund et al., 1997; Freund et al., 1998 and Rivolta et al., 2001; see also Section 2).

As the L and M gene array appears to have arisen from a gene duplication (Dulai et al., 1999), the L and M photopigment genes differ by only 2% at the nucleotide level in both exon and intron regions and contain repeat units of 39Kb of which ~15Kb is the visual pigment gene segment and ~24Kb is intergenic sequence. The similarity between L and M repeat units predisposes the tandem array to unequal homologous recombination. If crossing over occurs within the intergenic region then a gain/loss of one or more of the genes results, which accounts for the highly variable number of M genes in the normal trichromatic population: 25% have two retinal pigment genes in their L and M array, 50% have three, 20% carry four and 5% have five or more (Wolf et al., 1999). Recombination within the coding regions creates hybrid genes and most intragenic crossovers occur within introns (which are highly homologous, Shyue et al., 1994) due to their large size relative to the exons. These unequal crossovers produce hybrid genes that result in common abnormal colour vision disorders in males (Deeb et al., 1992). Figure 1.5 displays an example of how variations of

the L/M array arise due to crossover events. Some of the arrays depicted in Figure 1.5 will lead to a male suffering dichromacy, i.e. an absence of a single pigment class.

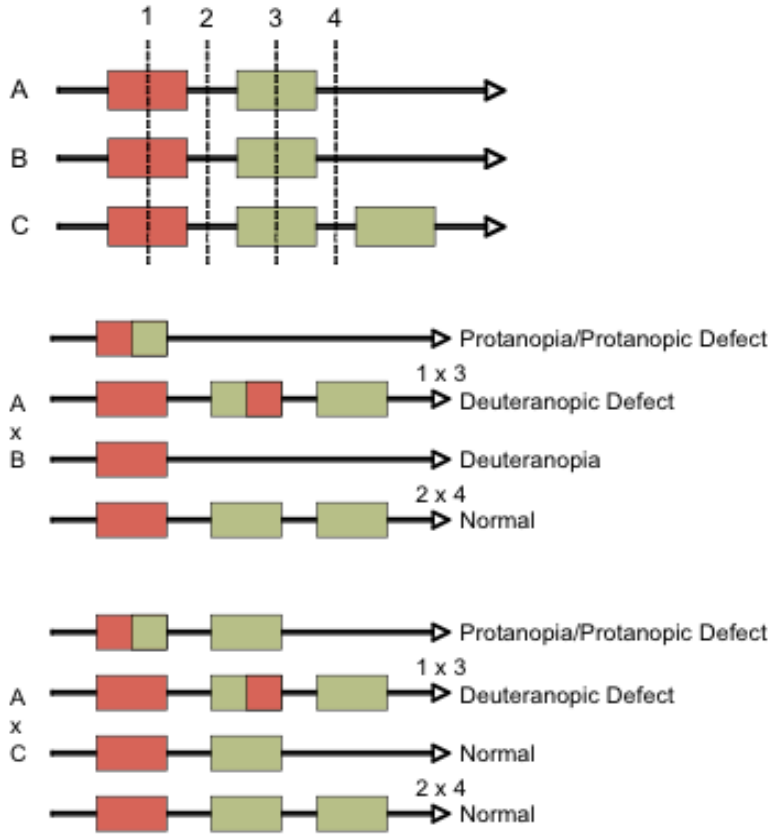


Figure 1.5 A schematic adapted from Nathans (1999). Red boxes indicate the sequence for the L gene and green boxes the sequence for M (exons and introns). Numbers 1 and 3 indicate intragenic crossover sites and 2 and 4 intergenic sites. Letters A-C represent different alleles of the array. The AxB grouping shows the arrays formed when unequal recombination occurs between breakpoints 1 and 3 or 2 and 4 if alleles A and B are present. The bottom grouping represents the arrays that may be produced following unequal recombination between alleles A and C at breakpoints 1 and 3 or 2 and 4. The hybrid L/M genes tend to produce an opsin with characteristics resembling M whereas the M/L hybrids tend to produce an opsin with L characteristics.

True dichromats can be classified as one of three types: protanopes, who lack functional L opsin; deuteranopes, who lack

functional M opsin; and tritanopes, who lack functional S opsin. There are also cases of anomalous trichromacy in which patients do not completely lack red or green vision, such individuals would be protanomolous or deuteranomolous, respectively. Nathans et al. (1986a) showed that these protan defects will arise when a male has an array consisting of an L/M hybrid gene followed by a normal M gene sequence. Such an individual may have complete protanopia or be protanomolous depending on how many exons of the hybrid are of the L opsin gene. Deutan defects will arise if an array consists of an L gene sequence followed by a hybrid M/L sequence (see Figure 1.5 for diagrammatic representations of these arrays).

The amino acid sequences of L and M cone opsins are ~96% identical and share ~42% identity with S-cone opsin and rod opsin (Nathans et al., 1986b). There are just 15 amino acid differences between L and M opsins (Figure 1.6), half of which are within the transmembrane region of the protein and therefore are likely to interact with the chromophore. Of these, three (Ser180Arg, Tyr277Phe and Thr285Ala) are known to contribute greatly to the majority of differences in spectral characteristics between the two opsins (Asenjo et al., 1994). If the seven residues Ser116Tyr, Ser180Ala, Iso230Thr, Ala233Ser, Tyr277Phe, Thr285Ala and Tyr309Phe are changed in the M pigment then the absorption spectrum becomes indistinguishable from an L pigment spectrum. Ser180Ala is a common polymorphism, males with L(Ser180) photopigment are more sensitive to red light than those with L(Ala180), as shown by colour-matching experiments (Winderickx et al., 1992b). The λ_{max} of L(Ser180) has been shown *in vivo* to be ~3nm longer than that of L(Ala180) (Sharpe et al., 1998).

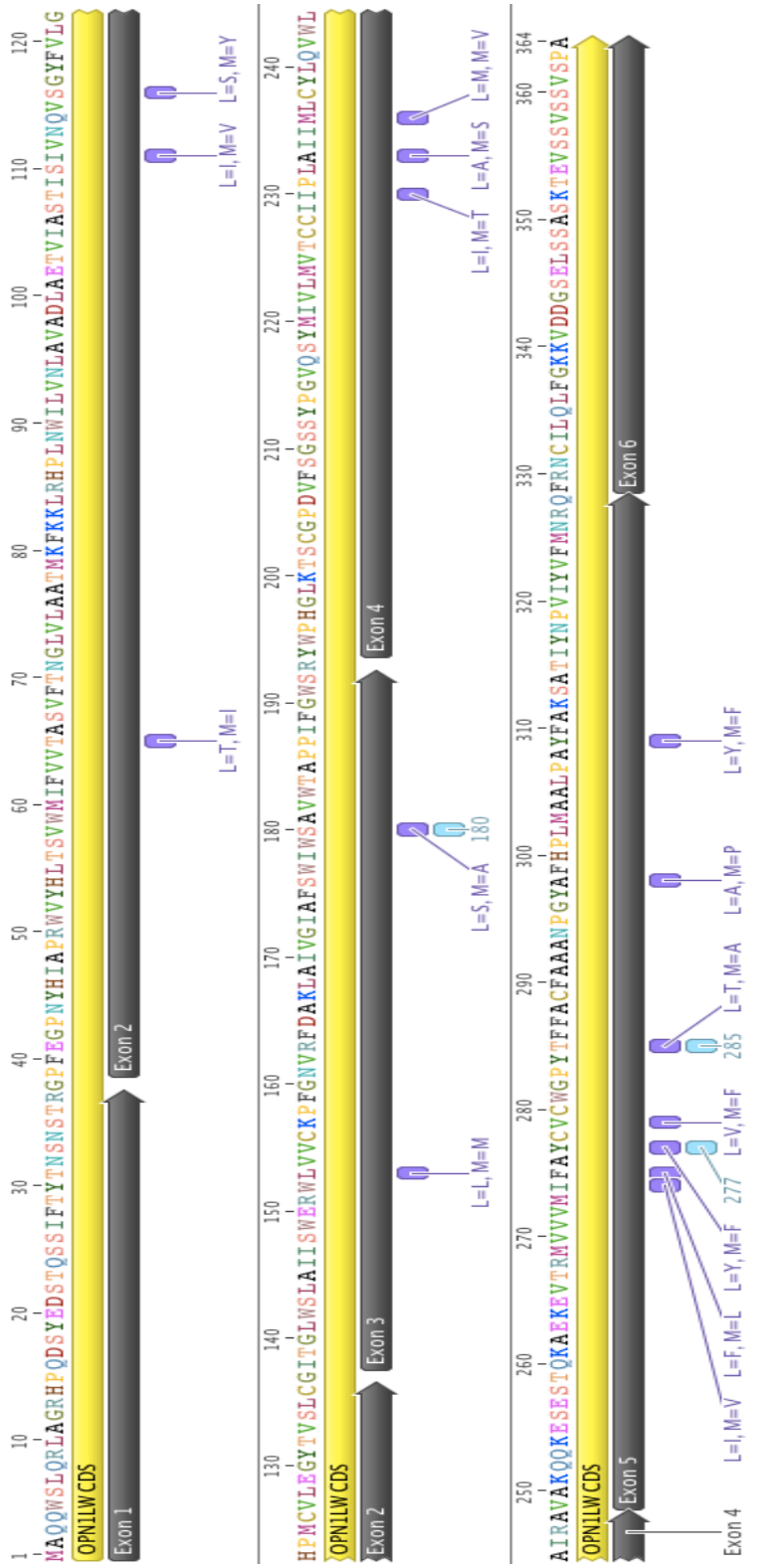


Figure 1.6. Image showing the amino acid sequence of L opsin with differences in M opsin highlighted.

When crossing over produces hybrid genes, the extent of the protan or deutan defects depends on the difference in the λ_{max} between the first two encoded pigments.

When Deeb et al. (1992) confirmed the array findings in protanopic and deuteranopic patients, they also reported a common point mutation in exon 4, nucleotide 609, that results in an amino acid change at position 203 of cysteine to arginine (Cys203Arg). Winderickx et al. (1992c) showed that if this missense mutation lies in one of the first two M genes of the array it leads to severe deuteranomaly. However, the Cys203Arg point mutation is not unique to these dichromats as it is present in 2% of the Caucasian population with normal colour vision. It is thought that unexpressed M genes in these individuals carry the mutation.

In summary, the L and M opsin genes are 98% identical at the nucleotide level and are present in a tandem array controlled by an LCR on the X chromosome. The high homology between both the gene and intergenic sequences gives rise to unequal recombination between the arrays which results in colour defects in males being fairly common. The 364 amino acid products of the two genes share a 96 % identity with only 15 amino acids differing, three of which contribute to the majority of the differences in the spectral characteristics of the two photopigments. However, though the three cone types are the basis for trichromacy in humans, retinal ganglion cells (RGCs) that respond in an opposing fashion following activation of the different cone classes, are the basis for colour opponency. This theory suggests that the visual system records differences between the responses of cone classes rather than each cone types individual response. Conway (2009) recently reviewed colour

opponency in which cone signals are processed by several classes of RGCs (Masland, 2001). Each neuron receives signals from a particular area of the retina, which corresponds to its visual/receptive field. Colour contrast is achieved from the comparison of the relative cone activations in adjacent patches across the visual space (Hurlbert & Wolf, 2004; Vladusich, 2007).

1.2.5 Phototransduction

The first step in the process of the transduction of light energy into membrane potential is the absorption of photons by the light-sensitive pigments in the discs of the photoreceptor outer segments. In the process of rod phototransduction this is achieved by rhodopsin, which is located in the membranes of rod discs. This photopigment contains seven hydrophobic alpha-helical transmembrane domains linked by hydrophilic loops (Trumpp-Kallmeyer et al., 1992). The seven alpha-helical segments form a pocket in which the chromophore, 11-*cis* retinal, sits and covalently bonds via a protonated Schiff base linkage to a specific lysine residue (Lys296) in helix six (Hargrave, 2001). Following light absorption the chromophore changes to the all-*trans* form, which converts the rhodopsin to metarhodopsin. Metarhodopsin I (Meta I) is initially formed and contains the protonated Schiff base but is quickly converted to metarhodopsin II (Meta II), in which the Schiff base is deprotonated and in this form the molecule is more sensitive to hydrolysis (Lamb and Pugh, 2004). Meta II is the active form that stimulates the heterotrimeric G protein transducin. In its inactive state, transducin is bound to guanosine diphosphate (GDP) but Meta II catalyses the substitution of guanosine triphosphate (GTP) for GDP. This occurs on the transducin α subunit, which dissociates from Meta II and the other two transducin subunits, β and γ. Meta II catalyses around

500 such reactions before it is inactivated by the combined actions of rhodopsin kinase and retinal arrestin.

The GTP-linked α subunit of transducin activates cyclic guanosine monophosphate (cGMP) phosphodiesterase (PDE) by removal of the inhibitory phosphodiesterase γ subunits, allowing the α and β PDE subunits to hydrolyse cGMP. cGMP that is present in the rod cytoplasm in the absence of light is converted into GMP, which reduces the amount of cGMP in the cytosol and causes the cGMP-gated cation channels on the membrane of the rod cell to close, leading to hyperpolarisation of the photoreceptor cell membrane. Hyperpolarisation causes calcium channels on the inner segment to close and as a result, fewer neurotransmitter molecules are released from the synaptic terminal. Thus, information about the presence of light is relayed by a decrease in signal to the bipolar cells. To return the cell to its dark-adapted state the cGMP must be regenerated, this is done in several ways, one of which involves retinal guanylate cyclase-1 (retGC1). As intracellular Ca^{2+} levels fall, retGC1 is activated by Ca^{2+}-dependent guanylate cyclase activating protein (GCAP) to regenerate cGMP. Mutations in both retGC1 and GCAP have been identified and result in dysregulation of intracellular Ca^{2+} and cGMP levels, which is believed to lead to cell death (reviewed by Hunt et al., 2010). Mutations in *GUCY2D,* which encodes retGC1, are responsible for around 6% of LCA cases (Lotery et al., 2000), with mutations in this gene also responsible for cone-rod dystrophy (Kelsell et al., 1998 and Udar et al., 2003). These mutations reduce retGC1 sensitivity to increased cellular calcium levels, leading to failure of retGC1 to be inhibited by GCAP. Mutant GCAP (as in Payne et al., 1998) seems able to activate retGC1 in low Ca^{2+} concentrations but fails to inactivate it at high Ca^{2+} concentrations, resulting in constitutive activation of retGC1 in the photoreceptors.

The photopigment is rapidly inactivated and restored to the responsive state as it cannot signal the arrival of another photon of light until the all-*trans* retinoid is replaced by new 11-*cis* retinal. The isomerised retinoid is removed in a long series of reactions called the retinoid cycle (Section 1.3.1). In rods, the all-*trans* retinal is released from the opsin and taken out of the cell across the extracellular space to the RPE where it is converted to 11-*cis* retinal, which can recombine with the opsin. It has been suggested that cone visual pigment regeneration occurs outside the RPE and that the isomerised retinoid is processed in Müller cells (Mata et al., 2002). However, Kanan et al. (2008) recently showed in their *in vitro* studies that a Müller cell line did not provide the necessary enzymatic activity to complete the retinoid cycle and proposed that cone pigment regeneration still requires the RPE. If the cone-specific visual cycle occurs in the Müller cells then it may be that inter-photoreceptor retinol binding protein (IRBP) is important in cone opsin regeneration (Muniz et al., 2007). Parker et al. (2009) have provided evidence that IRBP could be responsible for transporting the retinoid from Müller cells to the cone inner segments where it can be oxidised to 11-*cis* retinal.

1.3 Retinal Pigment Epithelium

This is a single layer of cells lined in a regular array beneath the rods and cones and its role is to support the photoreceptors in various ways. Previously mentioned is the participation of the RPE in the light-dependent cycling and re-isomerisation of retinal from the all-*trans* state to the 11-*cis* state. This is important in the regeneration of photolysed visual pigments in the photoreceptor outer segments. In addition to this the RPE stores retinyl esters, which are needed in the retinoid cycle. Faults in the cycle are

known to cause inherited retinal diseases (Thompson and Gal, 2003; Section 2).

1.3.1 Retinoid Cycle

This process will be outlined using information taken from the comprehensive reviews by Lamb and Pugh (2004), Travis et al. (2007) and Kono et al. (2008). The cycle begins with an 11-*cis* retinal chromophore absorbing a photon of light and isomerising to all-*trans* retinal, which activates the phototransduction cascade (Section 1.2.5). The all-*trans* isoform is removed from the photoreceptor outer segments in two ways: it can remain non-covalently bound to the opsin and in this state be reduced by all-*trans* retinol dehydrogenase (RDH) with the all-*trans* retinol produced following this reaction being released by the opsin, or: the all-*trans* retinal can be released from the opsin and form a condensation product, *N*-retinylidene-phosphatidylethanolamine (*N*-ret-PE), in the lipid membrane. This product gets flipped by ATP-binding cassette transporter (ABCA4) across the membrane into the cytoplasm where the all-*trans* retinal can be released, hydrolysed and then reduced by all-*trans*-RDH. In both pathways the reduced alcohol (all-*trans* retinol, also known as vitamin A) is chaperoned across the inter-photoreceptor matrix (IPM) by inter-photoreceptor retinol binding protein (IRBP) to the RPE. Mutations in *IRBP* are an infrequent cause of RP (Valverde et al., 1998 and den Hollander et al., 2009). Within the RPE, vitamin A is converted into 11-*cis* retinaldehyde in the following manner: all-*trans* retinol is chaperoned in the cytoplasm by cellular retinol binding protein (CRBP) before being esterified by lecithin retinol acyl transferase (LRAT). RPE65 chaperones the all-*trans* retinyl ester (which can also be stored) and retinyl ester isomerohydrolase then mediates the isomerisation of this

molecule to 11-*cis* retinol and the energy from the hydrolysis of the ester bond is thought to drive the isomerisation reaction. The protein RPE65 is important for this isomerisation and initially it was thought not to have any intrinsic isomerase activity (Redmond et al., 1998), however, more recently Nikolaeva et al. (2009) have shown it to be an isomerase. Mutations in *RPE65* are responsible for around 2% of autosomal recessive RP cases and 16% of LCA cases (Morimura et al., 1998). Following isomerisation, the alcohol is oxidised by 11-*cis* retinol dehydrogenase to the aldehyde form (11-*cis* retinal), a reaction accelerated by cellular retinaldehyde binding protein (CRALBP, Saari et al, 2001), which also chaperones the 11-*cis* retinal in the RPE cytoplasm. Alternatively, the 11-*cis* retinol can be esterified and stored as 11-*cis* retinyl esters in the RPE. The 11-*cis*-retinal diffuses across the sub-retinal space to the photoreceptor outer segment where it can again bond to an opsin protein to form visual pigment.

1.3.2 Other Roles of the RPE

Another main role of the RPE that has been previously mentioned is the engulfment and degradation of distal outer segments to counter the constant renewal of the outer segments. Each RPE cell contacts 50 to 100 photoreceptor outer segments and engulfs a large amount of these shed segments, which constitutes a high level of phagocytic activity especially in light of the fact that the RPE cells themselves continue throughout life with little or no turnover. A significant consequence of this activity is a progressive build-up of lipofuscin (a residue of outer segment breakdown) and it is thought this molecule could be a factor that predisposes the RPE to disease. Increased lipofuscin is thought to reflect increased outer segment turnover or the inability of the RPE to process the outer segment debris.

Cells of the RPE also absorb excess light by way of melanin granules and transport oxygen, nutrients and cellular wastes between the photoreceptors and the choroid. Separating the RPE layer from the blood vessels of the choroid is Bruch's membrane, a thin structure of collagen and elastic fibres.

The choroid is a vascular layer of the eye that provides nourishment to the outer layers of the retina and is composed of different structures: the choriocapillaris, Haller's layer, Sattler's layer and the suprachoroid. The choriocapillaris is a densely branching capillary network of the choroidal circulation that supplies nutrients and oxygen to the photoreceptors.

1.4 Summary

The retina is formed from various cell types that communicate with each other to produce a signal sent via the optic nerve to the brain, which produces a visual image. It is important to have a basic understanding of the physiology of the eye and the molecular biology of the visual process in order to better understand how faults in any part of the structure and/or visual pathways may lead to impaired vision. In such a small area the eye packs in millions of highly sensitive cells that are interacting in different ways and not simply sending signals to the brain but rather combining, superimposing and collecting a variety of information that is then forwarded for processing. If even a few cubic millimetres of the retina is damaged it can greatly reduce an individuals ability to respond to visual stimuli. Due to the complex structure and numerous biological processes occurring in this organ, a small error in any part of the visual system may have a detrimental effect on the sight of an individual. In this project attempts will be made to establish the genetic cause of a number

of retinal dystrophies, which will hopefully lead to a better understanding of the complex molecular pathways involved in retinal function and dysfunction.

2. INHERITED RETINAL DYSTROPHIES

An Overview

2.1 Retinal Degeneration

Inherited retinal dystrophies are a heterogeneous group of disorders, which result in degeneration of the retina. This may begin with a mutation in a gene that causes a problem in one particular cell type that then leads to the malfunctioning of that cell. As processes within the cell are disrupted this triggers apoptosis and therefore death of the cell. Depending on the genetic cause, cell death may be confined to one particular cell type, for example in cone dystrophies. In other retinal dystrophies the genetic fault will lead to apoptosis of a particular cell type but then loss of that cell type will trigger apoptosis of others, as in retinitis pigmentosa (RP) where primary loss of rods then leads to loss of cones, as cone survival is dependent on rods. The genetic cause therefore determines whether vision in the periphery (as in RP) or central vision (as in macular and cone dystrophies) is lost. Cell death can occur in all layers of the retina, including the neuronal cells of the inner and outer retina and the RPE. Some disorders involve not only loss of the neuronal cells and RPE but also the vasculature and formation of sub-retinal neovascular membranes (SRNVM), result in an unstable phenotype.

Inherited retinal dystrophies have been shown to be caused by mutations in over 250 different genes (listed online at the retinal disease database: http://www.sph.uth.tmc.edu/RetNet/) and show a range of inheritance patterns. For example, RP affects 1 in 3,500 people worldwide and autosomal dominant, autosomal

recessive, X-linked and sporadic patterns of inheritance have all been reported (Wang et al., 2005). In addition to this, a retinal dystrophy that mimics RP has been seen in association with mutations of mitochondrial DNA (Holt et al., 1990). RP is a degenerative process that primarily affects the rod photoreceptors and is characterised by night blindness, progressive loss of periphery vision with an eventual complete loss of vision. Central vision and macular function are generally preserved until the later stages of disease when cone loss occurs. At least 36 genes have been associated with this disorder but despite this the genetic cause of over 50% of RP cases have yet to be identified. Rhodopsin alone has had more than 100 mutations associated with RP and mutations in the *RHO* gene account for around 25% of autosomal dominant RP cases in the US. Other genes in which mutations have been identified as causing RP are shown in Table 2.1.

The rod-cone dystrophy leber congenital amaurosis (LCA) presents from birth or within the first few months of life and generally shows an autosomal recessive pattern of inheritance. LCA accounts for ~5% of retinal disease cases and is characterised by visual loss at birth, minimal or absent ERG, nystagmus and a variety of fundus changes. Fifteen genes/loci have so far been associated with this disorder and can be seen in Table 2.2.

In cone-rod dystrophies vision loss is caused by initial deterioration of cones followed by loss of rods. So far around twelve genes have been identified as being involved in this form of retinal degeneration and these are shown in Table 2.2.

Table 2.1 Genes in which mutations have been identified as causing forms of RP.

Disorder	Gene	Location	References	Other Retinal Diseases
Autosomal Dominant Retinitis Pigmentosa	*PRPF3*	1q21.2	Chakarova et al. (2002)	
	SEMA4A	1q22	Abid et al. (2006)	
	BRR2	2q11.2	Zhao et al. (2009)	
	RHO	3q22.1	Sung et al. (1991)	AR RP
	PRPH2	6p21.1	Boon et al. (2008)	AD MD, digenic RP, VMD, CORD
	GUCA1B	6p21.1	Sato et al. (2004)	AD MD
	KLHL7	7p15.3	Friedman et al. (2009)	
	RP9	7q14.3	Maita et al. (2004)	
	IMPDH1	7q32.1	Bowne et al. (2006)	AD LCA
	RP1	8q12.1	Sullivan et al. (1999)	AR RP
	TOPORS	9p21.1	Chakarova et al. (2007)	
	ROM1	11q12.3	Sakuma et al. (1995)	Digenic RP (Kajiwara et al., 1994)
	NRL	14q11.2	Bessant et al. (1999)	
	PRPF8	17p13.3	McKie et al. (2001)	
	FSCN2	17q25.3	Wada et al. (2001)	AD MD
	CRX	19q13.32	Sohocki et al. (1998)	CORD, LCA
	PRPF31	19q13.42	Deery et al. (2002)	
	RPGR	Xp11.4	Buraczynska et al. (1997)	XCORD
Autosomal Recessive Retinitis Pigmentosa	*RPE65*	1p31.2	Morimura et al. (1998)	AR LCA
	ABCA4	1p22.1	Cremers et al. (1998)	STGD1, FFM, AR MD, CORD
	CRB1	1q31.3	den Hollander et al. (1998)	AR LCA
	MERTK	2q13	Gal et al. (2000)	CORD
	CERKL	2q31.3	Tuson et al. (2004)	CORD
	RHO	3q22.1	Azam et al. (2009)	AD RP
	PDE6B	4p16.3	Bayés et al. (1995)	
	PROM1	4p15.32	Permanyer et al. (2010)	MCDR2, STGD4, RP, CORD
	CNGA1	4p12	Dryja et al. (1995)	
	PDE6A	5q33.1	Huang et al. (1995)	
	TULP1	6p21.31	Banerjee et al. (1998)	AR LCA
	EYS	6q12	Abd El-Aziz et al. (2008)	
	RP1	8q12.1	Khaliq et al. (2005)	AD RP
	RBP3	10q11.22	den Hollander et al. (2009)	
	CDHR1	10q23.1	Bolz et al. (2005)	
	RGR	10q23.1	Morimura et al. (1999)	
	SPATA7	14q31.3	Wang et al. (2009)	LCA
	RLBP1	15q26.1	Maw et al. (1997)	
	CNGB1	16q13	Bareil et al. (2001)	
	IDH38	20p13	Hartong et al. (2008)	
	RPGR	Xp11.4	Buraczynska et al. (1997)	XCORD

Table 2.2 Genes in which mutations have been identified as causing various forms of inherited retinal dystrophy.

Disorder	Gene	Location	Reference	Other Retinal Diseases
Leber Congenital Amaurosis	*RPE65*	1p31.2	Morimura et al. (1998)	AR RP
	CRB1	1q31.3	den Hollander et al. (2004a)	AR RP
	RD3	1q32.3	Friedman et al. (2006)	
	TULP1	6p21.31	Hanein et al. (2004)	AR RP
	LCA5	6q14.1	Mohamed et al. (2003)	
	IMPDH1	7q32.1	Bowne et al. (2006)	AD RP
	RPGRIP1	14q11.2	Dryja et al. (2001)	CORD
	OTX2	14q22.3	Henderson et al. (2009)	
	RDH12	14q24.1	Perrault et al. (2004)	
	SPATA7	14q31.3	Wang et al. (2009)	AR RP
	GUCY2D	17p13.1	Lotery et al. (2000)	CORD
	AIPL1	17p13.2	Sohocki et al. (2000)	CORD
	CRX	19q13.32	Swaroop et a. (1999)	AD RP, CORD
Cone and Cone-Rod Dystrophies	*ABCA4*	1p22.1	Maugeri et al. (2000)	STGD1, FFM, AR RP, AR MD
	SEMA4A	1q22	Abid et al. (2006)	AD RP
	MERTK	2q13	Mackay et al. (2010)	AR RP
	CERKL	2q31.3	Aleman et al. (2009)	AR RP
	PROM1	4p15.32	Pras et al. (2009)	MCDR2, STGD4, RP
	PRPH2	6p21.1	Sohocki et al. (2001)	
	GUCA1A	6p21.1	Payne et al. (1998)	
	RIM1	6q13	Johnson et al. (2003)	
	ADAM9	8p11.23	Parry et al. (2009)	
	PDE6C	10q23.33	Thiadens et al. (2009)	
	CACNA2D4	12p13.33	Wycisk et al. (2006)	
	RPGRIP1	14q11.2	Hameed et al. (2003)	AR LCA
	GUCY2D	17p13.1	Udor et al. (2003)	AR LCA
	AIPL1	17p13.2	Sohocki et al. (2000)	AR LCA
	PITPNM3	17p13.2	Köhn et al. (2007)	
	UNC119	17q11.2	Kobayashi et al. (2000)	
	RAX2	19p13.2	Wang et al. (2004)	AMD
	RPGR	Xp11.4	Yang et al. (2002)	XRP
	CACNA1F	Xp11.23	Jalkanen et al. (2006)	
Macular Dystrophies	*ABCA4*	1p22.1	STGD1: Martinez-Mir et al. (1998) BEM: Michaelides et al. (2007)	FFM, AR RP, CORD
	EFEMP1	2p16.1	Stone et al. (1999)	AD drusen
	PROM1	4p15.2	Yang et al. (2008)	RP, CORD
	MCDR3	5p15.33p.13.1	Michaelides et al. (2003b?)	
	BSMD	5q21.2-q33.2	den Hollander et al. (2004b?)	
	PRPH2	6p21.1	BSMD: Nichols et al. (1993) AVMD: Wells et al. (1993) Best: Boon et al. (2007)	AD RP, digenic RP, CORD
	GUCA1B	6p21.1	Sato et al. (2004)	AD RP
	MCDR1/PBCRA	6q14-q16.2	MCDR1: Yang et al. (2007) PBCRA: Kelsell et al. (1995)	
	ELOVL4	6q14	Maugeri et al (2004)	
	BEST1	11q12.3	Marquardt et al. (1998)	
	MCDR4	14q11.2	Francis et al. (2003)	
	FSCN2	17q25.3	Wada et al. (2003)	AD RP
	MCDR5	19q13.31-q13.32	Yang et al. (2006)	
	TIMP3	22q12.3	Weber et al. (1994)	

2.2 Macular Dystrophies

An interesting group of inherited retinal dystrophies are those that specifically affect the macula. This is the area of the eye that surrounds the fovea and permits the greatest visual acuity. It is not known why there are disorders that specifically affect this region and defining the macula and its functions will help lead to a better understanding of this.

Inherited macular diseases lead to central vision loss and atrophy of the macula and underlying RPE. There are numerous inherited macular dystrophies, many with similar pathology to AMD and that show a range of inheritance patterns. The genetic causes of macular dystrophies have been reviewed by Michaelides et al. (2003a), Voo and Small (2004) and Mellough et al. (2009).

2.2.1 Stargardt Disease

This is the most common form of hereditary macular degeneration and accounts for around 7% of all macular dystrophies with 90-95% of these presentations in the form of STGD1. There are three types of STGD: autosomal recessive STGD1 (Figure 2.1a); autosomal dominant STGD-like macular dystrophy STGD2/3 (Figure 2.1b-c) and; autosomal dominant STGD-like disease STGD4.

STGD1 is a recessive form of juvenile macular degeneration and was first described by Karl Stargardt in 1909. The disorder is characterised by a progressive loss of central vision with onset occurring in the first or second decade of life. Fundus flavimaculatus (FFM) is a retinal disorder with clinical features very similar to those of STGD1 and is considered to be a late-

onset, slow progressive form of Stargardt's disease (Noble and Carr, 1979; Gelisken and Laey, 1985).

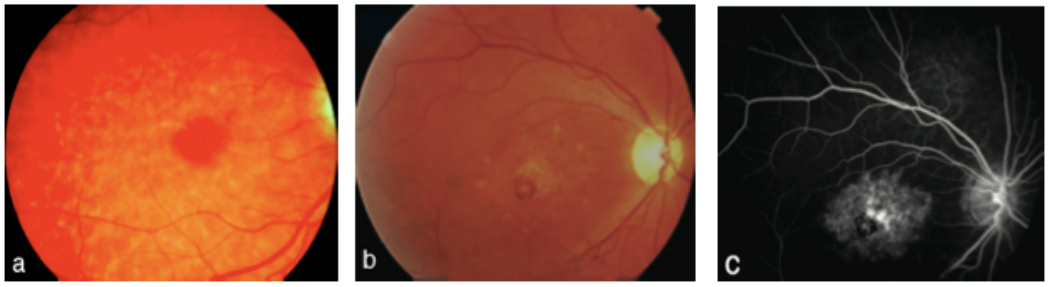


Figure 2.1 a) Fundus image taken from Michaelides et al. (2003a) of a typical patient with STGD; b) fundus image taken from an individual with Stargardt-like dystrophy caused by a Tyr2700X mutation in ELOVL4 and c) fluorescein angiography of the same individual as in (b). Images (b) and (c) are taken from Maugeri et al. (2004).

Both STGD1 and FFM are known to be caused by mutations in *ABCA4*, which encodes ATP-binding cassette transporter. This plays an important role in the recycling of retinoid and knockout mice lacking a functional *abca4* gene exhibit abnormalities in their visual cycle (Weng et al., 1991). Mutant ABCA4 leads to the condensation product *N*-ret-PE (Setion1.3.1) remaining inside the disc where it begins to form other intermediates. When these discs get phagocytosed by the RPE, the intermediates are converted to *N*-retinylidene-*N*-retinylethanolamine (A2E), a component of lipofuscin (Section 1.3.2). Mata et al. (2000) have shown that A2E levels are elevated in the RPE of abca4 knockout mice. This product has adverse effects (reviewed by Sparrow et al., 2005) such as interfering with lysosomal functions (Holtz et al., 1999), resulting in failure of the RPE to completely digest phospholipids (Finnemann et al., 2002). Over 500 mutations in *ABCA4* are known to cause Stargardt's disease and these can occur as frameshift, splice-site, deletions/insertions and missense forms.

Mutations in *ABCA4* are also responsible for autosomal recessive RP (Martinez-Mir et al., 1998 and Nasonkin et al., 1998), CORD (Cremers et al., 1998 and Maugeri et al., 1998) and FFM (Rozet et al., 1998). Michaelides et al. (2007) have also shown that *ABCA4* mutations occur in patients with BEM.

The phenotype of STGD3 is very similar to that of STGD1 and based on fundus examination alone it is difficult to distinguish between the two types. However, they do differ at the DNA level for whereas variations in *ABCA4* result in STGD1, mutations in the gene elongation of very long chain fatty acids-like 4 (*ELOVL4*) result in STGD3 (Maugeri et al., 2004). Zhang et al. (2001) identified a five base pair deletion (c.797-811delAACTT) in exon 6 of this gene, which causes a frameshift mutation and premature termination of the encoded protein. A second nucleotide variation in *ELOVL4* associated with STGD3 was identified by Bernstein et al. (2001), which was also found to cause butterfly macular dystrophy and bull's-eye maculopathy. This variation consists of two single base pair deletions separated by four nucleotides at the same location as the 5 bp deletion and which also results in a frameshift mutation and truncation of the ELOVL4 protein. In rhesus monkey and mouse retina, expression of this protein was found exclusively in rod and cone photoreceptors (Zhang et al., 2001). However, the specific role of this protein in the retina is unknown and thus the mechanism by which the deletions cause disease is currently unclear (review of STGD3 pathology: Vasireddy et al., 2010).

STGD4 exhibits a phenotype very similar to STGD1 and STGD3. Kniazeva et al. (1999) characterised this disorder and excluded linkage to either of the STGD1 and STGD3 loci and found it associated to 4p. Michaelides et al. (2003a) reported on a

missense mutation in *PROM1* that co-segregates with STGD4, mutations in which also cause RP and CORD. *PROM1* encodes human prominin (mouse)-like 1 protein, a member of the prominin family of 5-transmembrane domain proteins that is expressed in retinoblastoma cell lines and adult retina (Jászai et al., 2007). Further details of this protein will be discussed in Section 10 in relation to autosomal dominant Bull's-eye macular dystrophy (MCDR2).

2.2.2 Other Macular Dystrophies

Other macular dystrophies include the pattern dystrophies, which are a group of inherited disorders of the RPE that show yellow, orange or grey deposits within and under the RPE at the macula in a variety of distributions (Musarella, 2001). Butterfly macular dystrophy (BMD) is one form of distribution and mutations in the *RDS* gene are the predominant cause of the disease state with a second locus for BMD linked to 5q21.1-q33.2. Most mutations in the *RDS* gene associated with pattern dystrophies are missense mutations in the intra-discal D2 loop of the peripherin protein. This area is important for the tetrameric assembly of peripherin/Rds dimers (Goldberg and Molday, 1996) and heterotetrameric complex formation with the homologous protein ROM1 (Goldberg et al., 1995). Formation of these dimers is required for correct targeting and incorporation into newly formed rod outer segment disc membranes (Loewen et al., 2003). Mutations in *RDS* have previously been described to cause autosomal dominant RP and CORD but they are also known to cause adult vitelliform macular dystrophy (AVMD) and multifocal vitelliform macular dystrophy (Wells et al., 1993; Boon et al., 2007). Multifocal vitelliform macular dystrophy, better known as Best disease, is often confused with AVMD. Best disease is clinically heterogenous with the most prominent cause being

mutations in bestrophin-1 (*BEST1/VMD2*, Marquardt et al., 1998). Numerous mutations in *BEST1* have been identified but other genes associated with this phenotype are yet to be found. Biallelic mutations in *BEST1* have been shown to cause autosomal recessive bestrophinopathy (Burgess et al., 2008) and mutations that disrupt splicing have been shown to cause autosomal dominant vitreoretinochoroidopathy (ADVRC, Yardley et al., 2004).

Generally the pattern dystrophies do not cause significant visual loss. In contrast to this, the macular disorder Doyne honeycomb retinal dystrophy results in patients becoming legally blind, which occurs due to macular atrophy and, occasionally, sub-retinal neaovascular membrane (SRNVM) development. The small round yellow-white deposits that appear under the RPE of the macula and around the optic disc that are characteristic of this disorder, appear in early adult life. Despite this, good visual acuity is maintained until the fifth decade of life, after which, vision may be lost due to macular atrophy and subretinal neovascular membrane (SRNVM). A single mutation (Arg345Trp) in the gene encoding EGF-containing fibulin-like extracellular matrix protein 1 (*EFEMP1*) has been identified in individuals exhibiting Doyne honeycomb retinal dystrophy (Stone et al., 1999). The mutation Arg345Trp was also associated with Malattia Leventinese, which is an autosomal dominant macular disorder characterised by yellow-white drusen deposits that accumulate beneath the RPE. Aberrant accumulation of EFEMP1 also underlies drusen formation in AMD (Marmorstein et al., 2002).

Other genes in which mutations have been identified as causing autosomal dominant macular dystrophies include the previously mentioned *FSCN2* (Wada et al., 2003), mutations in which also cause autosomal dominant RP (Wada et al., 2001). Similarly,

GUCA1B mutations were previously described as causing autosomal dominant RP and mutations in this gene have also been associated with macular degeneration (Sato et al., 2004).

Sorsby's fundus dystrophy is a macular dystrophy that results in visual loss from SRNVM (Weber et al., 1994b). Mutations in exon 5 of the tissue inhibitor of metalloproteinase-3 gene (*TIMP3*) have been identified as being responsible for Sorsby's fundus dystrophy (Weber et al., 1994a).

Other autosomal dominant macular dystrophy loci have been identified at chromosome 5p15.33-p13.1 (MCDR3), 6q14.1-q16.2 (MCDR1/PBCRA), 14q11.2 (MCDR4) and 19q13.31-q13.32 (MCDR5). The first three of these loci will be investigated in this project.

2.3 Complex Disorders – Age-Related Macular Degeneration

Of the forms of macular degeneration, AMD is the most common, affecting ~30% of adults aged 75 or over in the US (Friedman et al., 2004). AMD is a complex disorder meaning it is influenced by numerous risk factors including age, cigarette smoking and hypertension (Hyman and Neborsky, 2002). Epidemiological studies have shown that family members are at increased risk of the disease (Smith and Mitchell, 1998) and twin and family-based studies provide evidence for a genetic basis (Seddon et al., 2003). For complex disorders such as AMD in order to find genetic factors relevant to this disorder, twin-based and association studies are required. Whereas the dominant and recessive disorders previously covered are the result of mutations passed down from one generation to the next, the genetic cause

identification can be identified through linkage studies on affected families. For complex disorders such analysis cannot be undertaken as a genetic change is not the sole cause of the disorder but is instead a contributing factor and so may not occur in all affecteds. However, twin and association studies allow for mutations to be identified that are contributors to the development of the disease state. Many groups have identified risk loci in recent years and looked for mutations in genes such *EFEMP1, RDS, BEST1* and *TIMP3*, which are known to be involved in macular degeneration (Section 2.2.2). No mutations in these genes were associated with AMD, as reviewed by Patel et al. (2008). Many genes involved in the immune system have been implicated in AMD and in particular, complement components. The most important genetic associations in AMD involved complement factor H (CFH, Hageman et al., 2005). Following the association of CFH with AMD, other complement factors were investigated. CFB and C2 screenings showed that 74% of AMD subjects lacked a protective variant of these genes found in 56% of subjects without AMD (Gold et al., 2006). A C3 variant has also been associated with AMD by Yates et al. (2007). Other genes associated with AMD are listed in Table 2.3.

Table 2.3 Genes associated with AMD.

Disorder	Gene	Location	References
Age-Related Macular Degeneration	*HMCN1/FBLN6*	1q31.1	Fisher et al. (2007)
	CFH	1q31.3	Hageman et al. (2005)
	TLR3	4q35.1	Yang et al. (2008b)
	CFB & C2	6p21.33	Gold et al. (2006)
	TLR4	9q33.1	Zareparsi et al. (2005)
	ERCC6	10q11.23	Tuo et al. (2006)
	HTRA1	10q26.13	Yang et al. (2006)
	FBLN5	14q32.12	Stone et al. (2004)
	C3	19p13.3	Yates et al. (2007)

2.4 Systemic Disorders

Inherited retinal dystrophies can also be seen in combination with other inherited disorders. For example, RP may be secondary in aetiology and is known to be part of over 30 syndromes (Wang et al., 2005) and in such conditions is usually inherited in an autosomal recessive manner, as in the case of Usher syndrome and Bardet-Biedl syndrome. Usher syndrome is the most common form of deaf-blindness and is clinically and genetically heterogeneous with eight causative genes identified so far whose encoded proteins appear to be part of a dynamic complex present in hair cells of the inner ear and in photoreceptor cells of the retina (Kremer et al., 2006). Hearing loss for Usher syndrome is sensorineural and most severe for high frequencies. Based on the age of onset of RP, progression of hearing loss and the severity of the symptoms, this syndrome can be identified as one of three clinical subtypes (Smith et al., 1994). The genes associated with the Usher syndrome subtypes are reviewed by Reiners et al. (2006) and largely encode Usher proteins. The Usher proteins are essential in the morphogenesis of the stereocilia bundle in hair cells and in the calycal processes of photoreceptor cells in addition to being important in the synaptic processes of these cell types (Kremer et al., 2006). As the Usher interactome is involved in pathways common to the inner ear and retina, when it becomes disrupted Usher syndrome results. Other disorders that include an ocular and hearing phenotype include Stickler and Wolfram syndromes. Stickler syndrome is characterised by facial-skeletal abnormalities, sensorineural hearing loss, glaucoma, myopia and retinal detachment (Bennett and McMurray, 1990). Genes identified as causing this disorder include *COL11A1, COL9A1* and *COL2A1* (Richards et al., 1996; van Camp et al., 2006; Hoornaert et al., 2010). Wolfram syndrome is characterised by diabetes, optic atrophy and deafness (Cremers et al., 1977). The cause of

this disorder has been found to be mitochondrial deletions (Rötig et al., 1993) but the genes *WFS1* and *WFS2* have also been identified (Rigoli et al., 2010; Ajlouni et al., 2002).

Bardet-Biedl syndrome (BBS) is a rare developmental ciliopathy disorder that exhibits a range of clinical presentations, including retinal dystrophy, learning difficulties, obesity, polydactyly or syndactyly and renal dysgenesis. The genetics of BBS are quite complex and though initially identified as a recessive trait it appears to be a triallelic disorder, i.e. three mutant alleles at two loci are necessary for pathogenicity (Katsanis et al., 2001). More recently, Webb et al. (2009) described BBS as an autosomal recessive trait, genetically heterogenous ciliopathic condition caused by mutations in multiple genes. Other disorders combine macular dystrophies with physical abnormalities such as in the rare association of macular dystrophy and ectodermal dysplasia and ectrodactyly (EEM syndrome) and Sorsby syndrome. This latter disorder is not to be confused with Sorsby's fundus dystrophy (Section 2.4.3), for this is a rare, dominantly inherited combination of bilateral colobomas and apical dystrophy of the hands and feet (Thompson and Baraitser, 1988). It is not yet known what the genetic cause of this disorder is but Bacchelli et al. (2003) have shown that it is unlikely to be due to mutations in *ROR2*, which cause brachydactyly type B.

Whereas Sorsby syndrome is a dominant disorder in which patients exhibit an ocular phenotype in association with abnormalities of the hands and feet, EEM shows an autosomal recessive mode of inheritance. In 2005, Kjaer et al. reported that EEM appears to be the result of distinct homozygous mutations in *CDH3* (cadherin-3 precursor), a gene also responsible for congenital hypotrichosis with juvenile macular dystrophy (HJMD;

Indelman et al., 2007). Shimomura et al. (2008) determined *CDH3* to be a p63 target gene important in the development of the human limb bud and hair follicles.

2.5 Disorders Investigated in this Project

Knowing the genetic cause of an inherited disorder not only provides a better understanding of function in the retina but also clues to the pathology of a disorder. For example, in the case of STGD1, deposits in the RPE are visible by retinal imaging yet they are not directly due to the gene mutated. *ABCA4* mutations disrupt the normal function of the ABCA4 protein, which sits in the photoreceptor membrane discs. The ineffective ABCA4 causes disruption of the retinoid cycle, which leads to build up of lipofuscin in the RPE. This accumulation is toxic to the RPE cells and triggers apoptosis. Death of the RPE cells leads to death of nearby photoreceptors as they rely on the RPE for survival. So in the case of STGD1, identifying the genetic cause led to a better understanding of the disease pathology and, in particular, lipofuscin composition. It is also important to discover the genetic cause of disorders in order to develop treatments to either prevent the degeneration or alleviate symptoms (for example, anti-vascular endothelial growth factor treatments are used to prevent further blood vessels forming across the macula in the wet form of AMD). But for most inherited disorders the hope is that gene therapies can be developed to replace the faulty gene in the affected cell type, producing enough functional protein to recover correct function in the retina. The retina is a unique environment for gene therapy and advancements in this treatment method are occurring quickly. Gene therapy currently focuses on using the adeno-associated virus (AAV), which infects human cells but does not cause disease and results in a very mild immune response. In

2007, the first clinical trial of gene therapy using AAV-2 for LCA cases with *RPE65* mutations began at the UCL Institute for Ophthalmology and Moorfields Eye Hospital, with two further trials in the US. The results were promising and demonstrated that the technique is feasible, safe and can lead to improvement in visual function (Bainbridge et al., 2008 and Maguire et al., 2008). Current studies focus on ensuring the gene delivery is efficient and cell type specific by use of different viral capsids and promoters (Allocca et al., 2007). Candidates for gene therapies come from investigations into the genetic causes of diseases, as in this project. If the genetic problem can be found then we will not only gain further insight into the functions of the retina and the pathology of the disorder, but also a chance of developing a treatment for it. Macular dystrophies affect central vision and therefore have a distinct impact on the lives of individuals suffering from such disorders. If retina function, and subsequently vision, can be restored to patients this would have a distinct positive effect on their lives.

This project will investigate the genetic causes of a number of macular dystrophies in addition to a cone-based disorder, which are detailed below.

2.5.1 North Carolina Macular Dystrophy

North Carolina macular dystrophy (NCMD/MCDR1) was first described over 40 years ago yet the genetic cause continues to elude scientists. Unrelated NCMD families have been reported in the UK (Reichel et al., 1998), Germany (Pauleikhoff et al., 1997), France (Small et al., 1997), Belize (Rabb et al., 1998), America (Small et al., 1998) with a suspected NCMD family in Korea (Kim et al., 2006). Initial studies reporting the disease used various names to describe the disorder as a result of the variable

phenotype seen, including; central areolar pigment epithelial dystrophy (CAPED; Frank et al., 1974; Fetkenhour et al., 1976; Hermsen and Judisch, 1984); central pigment epithelial and choroidal degeneration (Leveille et al., 1982) and central retinal pigment epithelial dystrophy (Klein and Bresnick, 1982). The disorder was first described by Lefler et al. (1971) in the descendants of a large Irish family settled in North Carolina in the late eighteenth to early nineteenth century. The CAPED families identified in other early studies were later revealed to be descendants of the three founding brothers of the original North Carolina family (Small et al., 1992a) and thus a common name for the disorder was developed based on this origin. Small (1998) considered 13 unrelated NCMD families of various ethnic origins and found no genetic heterogeneity associated with this disorder and all reported MCDR1 families have been linked to the same region on chromosome 6q16.

MCDR1 shows some clinical and histopathological similarities to AMD; it is characterised by non-progressive atrophy of the RPE and overlying photoreceptor cells, abnormal accumulation of drusen, choroidal neovascularisation and loss of central vision. However, NCMD is an autosomal dominant, highly penetrant disorder with congenital or infantile onset, whereas AMD is a complex disorder that generally develops in the fifth decade of life (Section 2.5). The extent of the clinical manifestations observed in NCMD varies and forms the basis of the grades that define the severity of the phenotype. Small et al. (1992b) outlined three grades of NCMD based on fundoscopic appearances: in Grade I there are small drusen-like yellow deposits in the central macula (Figure 2.2a). Such patients are usually asymptomatic with normal or slightly subnormal visual acuity; in Grade II there are confluent drusen. Visual acuity is usually near normal unless

there is development of subretinal choroidal neovascular membranes (SRNVM; Figure 2.2b). Grade III denotes well-demarcated chorioretinal atrophy with hyper-pigmentation bordering the lesion (Figure 2.2c).

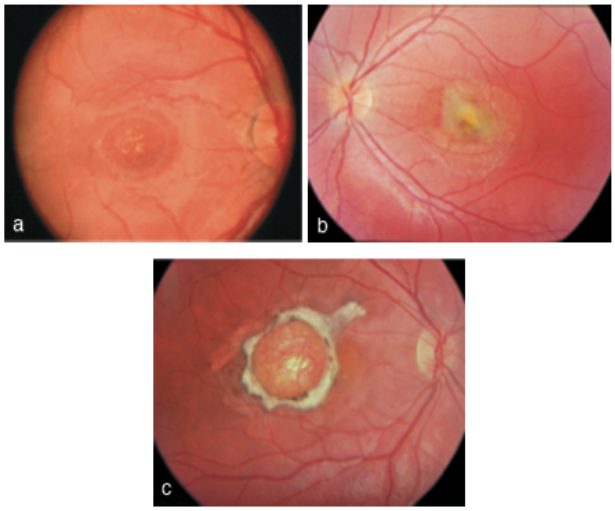


Figure 2.2 Fundus images taken from Yang et al. (2007): a) Grade I MCDR1; b) left eye of an MCDR1 patient with new choroidal neovascularisation (CNV) and c) Grade III MCDR1. See text for grade descriptions.

Generally the disease is stable except in individuals who develop SRNVM. The disease appears to be early-onset in all cases and Rhee et al. (2007) reported on a 3-year old with subfoveal neovascularisation that was first identified at 34 weeks with bilateral atrophy of the choroid and RPE confined to the macula.

Szlyk et al. (2005) performed comprehensive functional vision assessment of five individuals with NCMD and concluded that despite the phenotypic variability of this disorder, visual acuity of 20/200 or better appears to be maintained irrespective of the clinical grade of severity in all patients. The group also reported that visual acuity does not appear to significantly change over time and though the fundus appearance in NCMD is typically bilaterally symmetrical, characteristically one eye predominates in terms of visual function.

Many macular dystrophies exhibit an abnormal fundoscopic appearance confined to the macula yet present electrophysiological, psychophysiological or histophysiological evidence of more widespread photoreceptor and RPE dysfunction (Scullica and Falsini, 2001). However, MCDR1 psychophysiological and electrophysiological tests have revealed that normal peripheral retinal function is retained (Small, 1998). As MCDR1 appears to be macular-specific, the function of the gene involved in causing the disease will give insight into macular development and function.

Other retinal disorders have been linked to nearby chromosomal regions and in the case of progressive bifocal chorioretinal atrophy (PBCRA, Section 8), to an overlapping region. PBCRA, another developmental macular disorder, has a more severe phenotype than MCDR1 but it is possible that different mutations in the same gene cause both disorders. A retinal disease with phenotypic similarities to MCDR1 is dominant drusen and macular dystrophy, which has been linked to 6q14 (Kniazeva et al., 2000) but as with MCDR1, no specific genetic change has yet been identified. The previous genetic analysis conducted on MCDR1 families and the protocol used in this project are detailed in Chapter 5.

2.5.2 North Carolina-like Macular Dystrophy

The phenotype of this disorder is very similar to that of MCDR1 but linkage to 6q was excluded and linkage to 5p determined. So far, North Carolina-like macular dystrophy (MCDR3) has only been reported in a single family (Figure 2.3) in which the disease was characterised as resembling North Carolina macular dystrophy. Despite the similarities between the two disorders, the MCDR1 locus was excluded and the disorder mapped to

IV

V

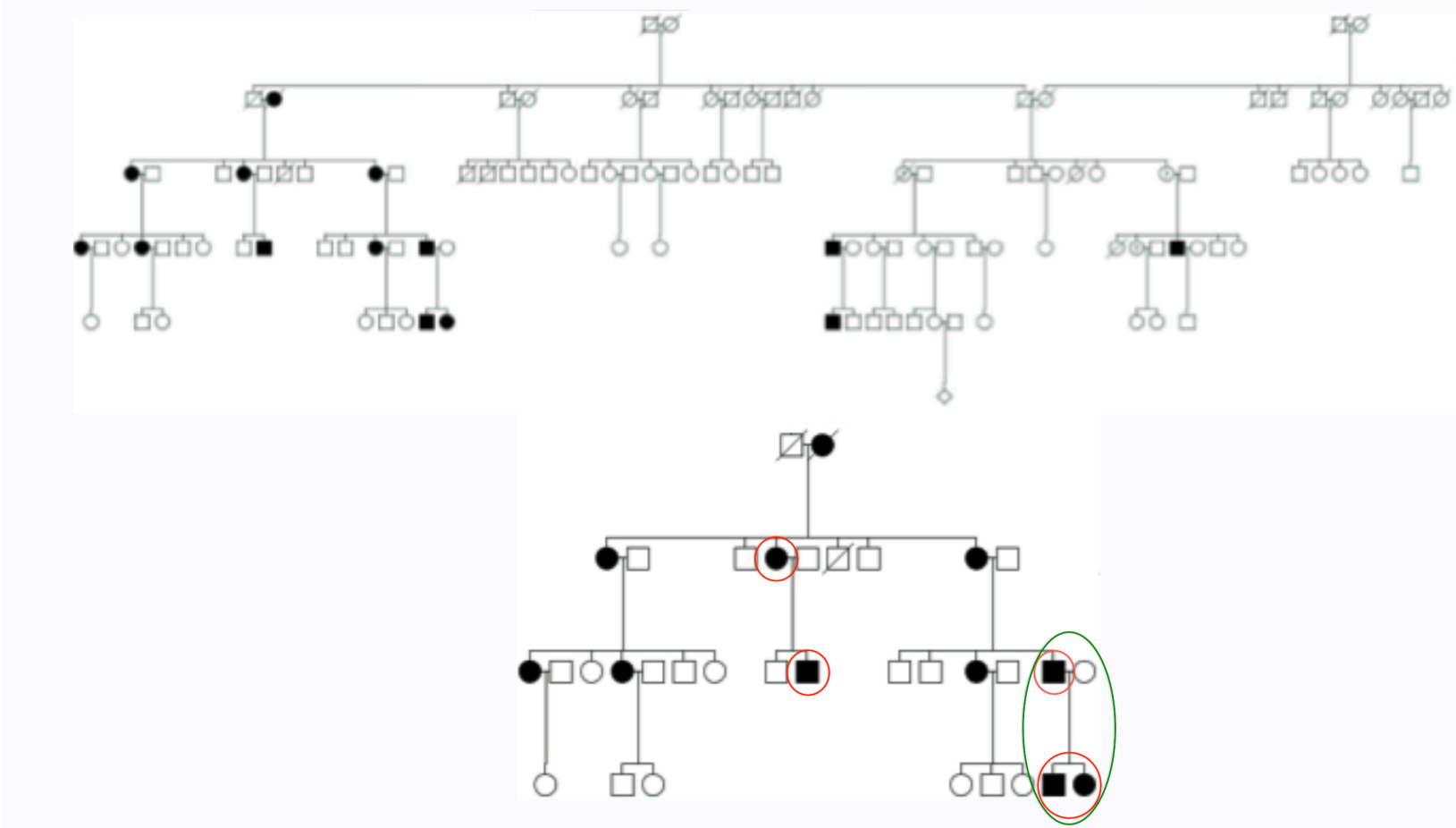


Figure 2.3 Six-generation family with autosomal dominant macular dystrophy with the branch of this family used in the study by Michaelides et al. (2003c) shown below. Circled in red are the samples run on the Affymetrix 250K *Sty* I chips and circled in green are those run on the Affymetrix 50K *Xba* I chips.

chromosome 5 (Michaelides et al., 2003c). The disease is characterised by an early age of onset (within the first five years of life) and vision is generally good despite the macular abnormalities seen, which range from multiple drusen-like deposits to focal atrophy and pigmentation (Figure 2.4 a-c).

The disease appears to be non-progressive though one individual (V:7) did show an increase in drusen-like deposits and retinal pigmentation over a 5-year period but there was no evidence of a change in the macular appearance during this time. As with MCDR1, the retinal phenotype varied amongst individual family members and all three grades of lesion characterised in MCDR1 were seen in the MCDR3 pedigree (Figure 2.4 a-c). Additionally, the visual field loss was not widespread and demonstrated only over the central macular lesions, suggesting this dystrophy is also localised to the macula. Two significant differences between MCDR1 and MCDR3 were apparent: mild abnormalities in colour vision in some of the MCDR3 affected individuals and evidence of disease progression, though this was only noted in a single case.

Whereas MCDR1 shares aspects of its phenotype with AMD, MCDR3 shows some differences to the complex disorder. In particular, concentric perifoveal areas of increased autofluorescence (AF) were found to correspond to the drusen-like deposits (Figure 2.4 a & b), whereas in AMD there is generally little association between the drusen and the AF (von Rückmann et al., 1997). The mechanism of drusen formation in both MCDR1 and MCDR3 is currently unclear but it is believed that identification of the causative genetic mutations of the two disorders will aid our understanding of this process. The genetics of MCDR3 are considered in Chapter 6.

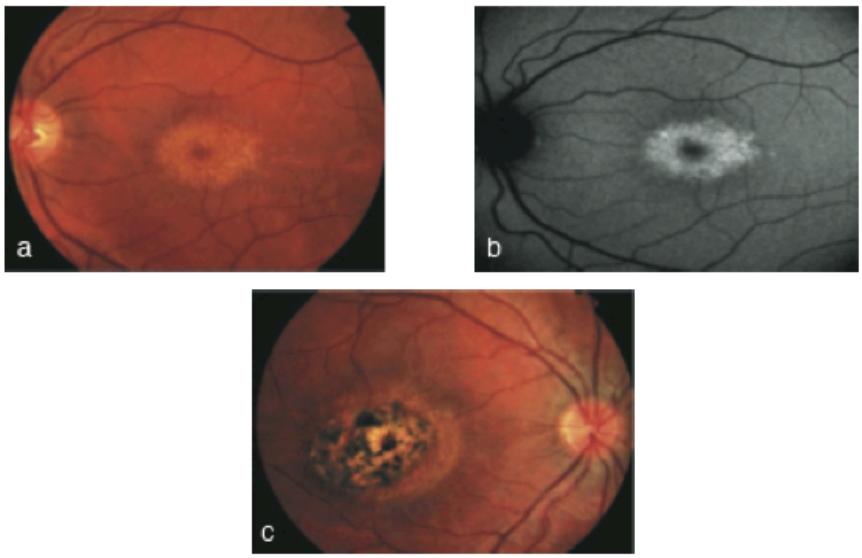


Figure 2.4 All images taken from Michaelides et al. (2003c): a) fundus photograph of patient IV:14; b) shows the fundus AF image from the same individual as in (a) and demonstrates increased AF at the macular that corresponds to the drusen-like deposits visible in (a) and c) fundus photograph of patient V:7 showing macular RPE atrophy and pigment clumping with surrounding drusen-like deposits.

2.5.3 North Carolina-like Macular Dystrophy with Progressive Sensorineural Hearing Loss

Again this disorder has a very similar phenotype to MCDR1 yet as with MCDR3, it does not link to 6q. Also as with the MCDR3 phenotype, this disorder has currently only been reported in a single publication by Francis et al. (2003) studying a four-generation family (Figure 2.5). This autosomal dominant inherited macular dystrophy is described as being clinically similar to MCDR1 but affected individuals also exhibit progressive adult-onset sensorineural hearing loss. The typical pattern of sensorineural hearing loss segregating with the ocular phenotype is progressive, bilateral, symmetrical and high frequency.

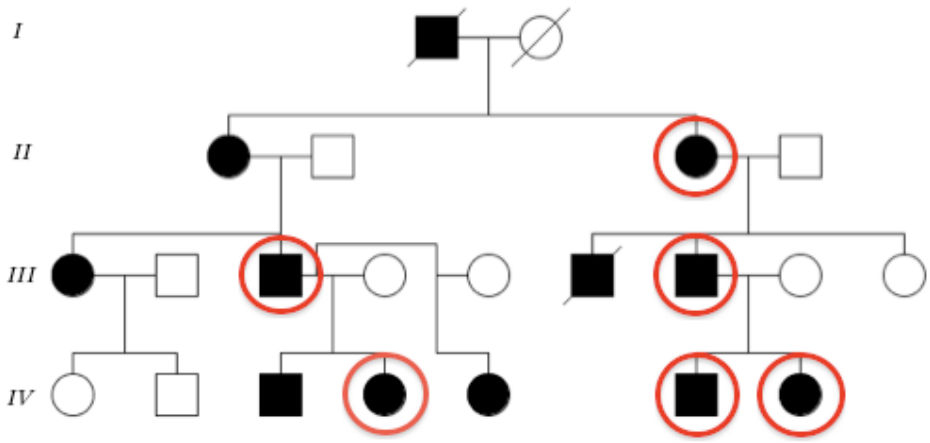


Figure 2.5 Four-generation pedigree of a family with autosomal dominant macular dystrophy and progressive sensorineural hearing loss. Circles indicate patient samples run on the Affymetrix 250K *Sty* I chips.

As seen in both MCDR1 and MCDR3 families, the ocular phenotype of the MCDR4 disorder varied among different family members. Some individuals have fine drusen-like deposits at the macula and pigmentary disturbance at the RPE (Figure 2.6a), a phenotype consistent with MCDR1 grade 1 appearances. Other individuals show a well-demarcated subfoveal area of chorioretinal atrophy with pigment hypertrophy bordered by fibrosis (Figure 2.6b), which is consistent with grade III MCDR1 lesions. As with both MCDR1 and MCDR3, the electrophysiological findings of the affected MCDR4 individuals were essentially normal, which implies the disorder is restricted to the macula region. The clinical appearances were non-progressive and evident soon after birth whilst the sensorineural hearing loss was progressive and typically became significant in the fourth decade of life.

Other retinal degenerations with associated hearing loss have been described, with the most common being Usher syndrome in

which the hearing defect is associated with retinitis pigmentosa (Kimberling and Möller, 1995, Section 2.4). This combination of retinal and hearing defects is unsurprising when considering the hair cells of the cochlear and retinal photoreceptors show structural similarities and have shared gene expression (Kremer et al., 2006). In Usher syndrome, both cone and rod photoreceptor systems are involved and the ocular disease is progressive, this contrasts to MCDR4, which appears to be a non-progressive macular-specific disorder with associated deafness. Similar disorders have been reported but appear to be caused by a mitochondrial defect (Harrison et al., 1997; Souied et al., 1998). Identification of the disease-causing gene will not only increase our understanding of the mechanisms of macular development but also of the ear. The genetics of MCDR4 are considered in Chapter 7.

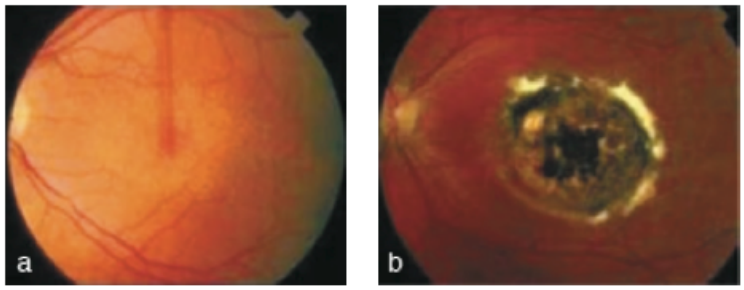


Figure 2.6 Fundus images taken from Francis et al. (2003): a) from patient III:1, showing drusen-like deposits and retinal epithelial atrophy centred on the macula and b) from patient III:3, showing subfoveal chorioretinal atrophy with pigment hypertrophy and fibrosis at the edge.

2.5.4 Progressive Bifocal Chorioretinal Atrophy

Progressive bifocal chorioretinal atrophy (PBCRA) was identified in a large five generation British family by Kelsell et al. (1995), Figure 2.7. Genetic linkage analysis was conducted and the PBCRA disease locus was subsequently mapped to chromosome 6q, which overlaps with the MCDR1 locus. The two disorders are, however,

clinically distinct. This congenital chorioretinal dystrophy was first reported by Douglas et al. in 1968 and the clinical features were described by Godley et al. in 1996. PBCRA is characterised by progressive macular and nasal atrophic lesions, nystagmus, myopia and poor vision. It has two distinct foci of atrophy: a temporal focus present at birth that then enlarges throughout life and a nasal focus that develops in the early years of life. Affected individuals show a reduction in colour vision and visual acuity though sight is never completely lost. Unlike MCDR1 and the NCMD-related disorders, PBCRA findings reflect widespread abnormality of photoreceptors and RPE as revealed by electroretinography (ERG) and electrooculography (EOG). Individuals also exhibit poor colour vision, which is a characteristic only seen in one of the other NCMD-related disorders, MCDR3. So, despite overlapping with the MCDR1 disease locus the two disorders present different phenotypes and therefore if the disorders are allelic, it seems likely that different mutations are involved. The genetic analysis previously conducted on this disorder will be considered in Chapter 8.

2.5.5 Split-Hand/Split-Foot Malformation with North Carolina Macular Dystrophy

In this project two families will be investigated, one English and one French (Figure 2.8), both exhibit a NCMD phenotype with additional bilateral macular 'colobomata' and skeletal abnormalities of the digits. The term macular coloboma, although commonly used, is actually a misnomer. Coloboma occur when there is a failure during development of the closure of the foetal fissure. Macular colobomas are not caused by such defects but represent a failure in the development of the fovea or macular atrophy. In the French family, some affected members have isolated macular 'colobomata' whilst others have 'colobomata' with

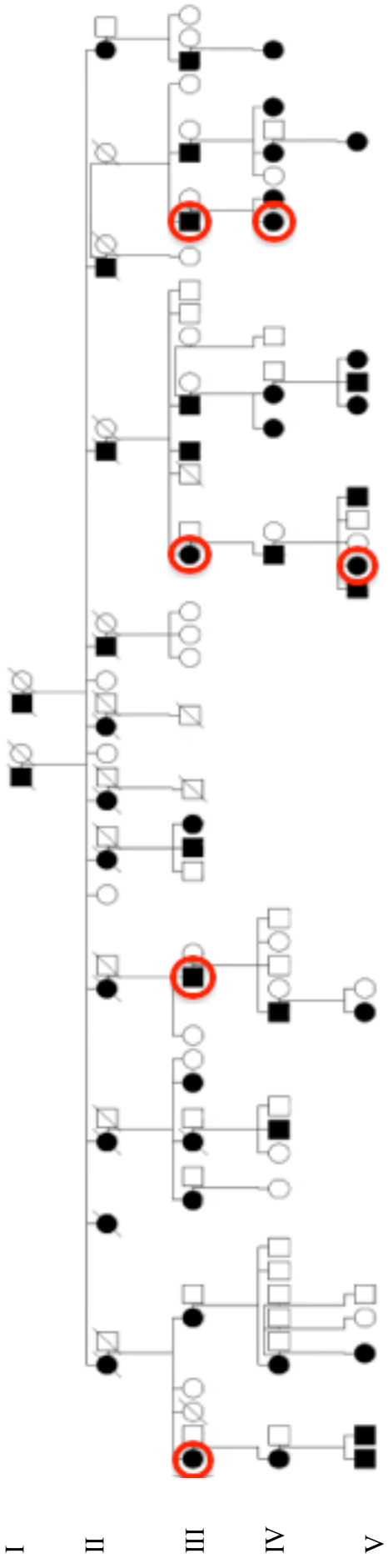


Figure 2.7 Pedigree of a five-generation family with progressive bifocal chorioretinal atrophy. Samples highlighted in red were analysed on a 250K Affymetrix *Sty* I chip.

digit anomalies (brachydactyly or SHFM). In both families the SHFM phenotype is typically variable amongst members of the same family whilst the presence of macular colobomata is fairly uniform amongst the affected individuals. For the French family (9.1b), individuals II:2, II:6 and V:1 exhibit macular coloboma; III:1, III:3, IV:1 and IV:3 exhibit coloboma and apical dystrophy with III:3 and IV:1 also exhibiting pachyonychia. Individual II:4 has macular dystrophy. Images in Figure 2.9.

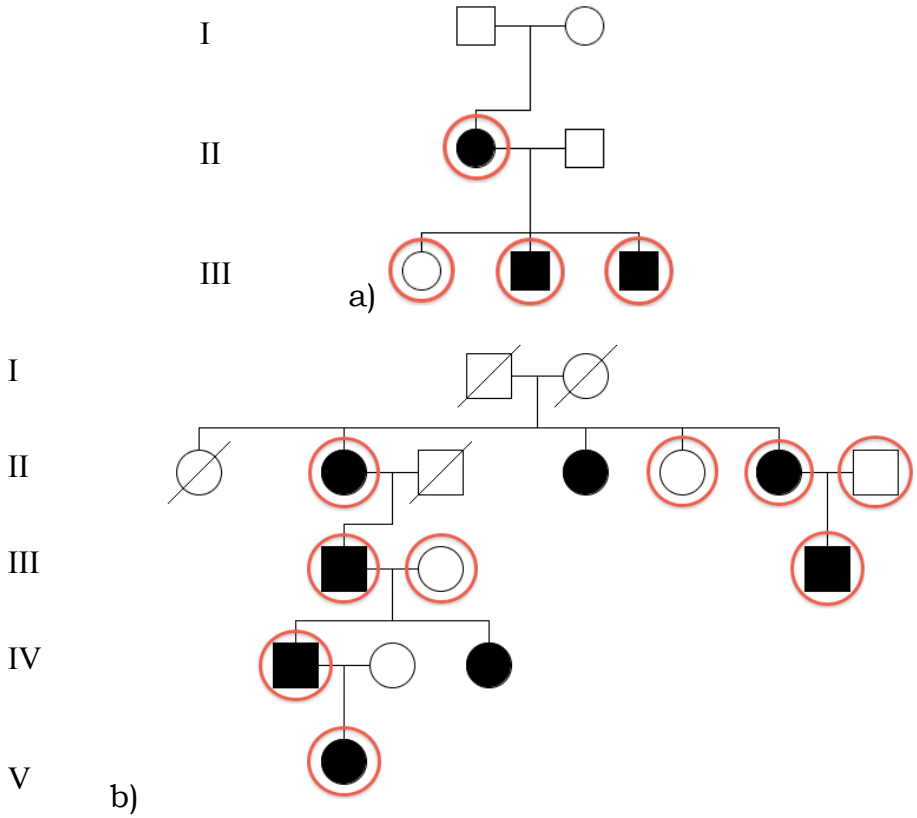
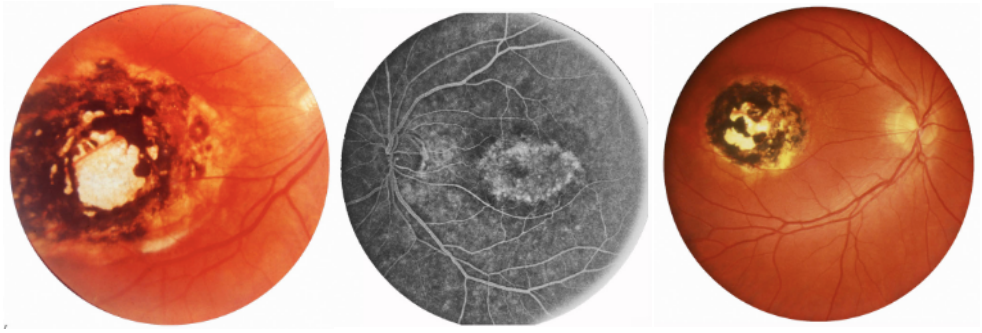


Figure 2.8 SHFM and NCMD pedigrees: a) a three-generation English family and b) a five-generation French family. Circled in red are samples run on the Affymetrix 250K SNP Chips.



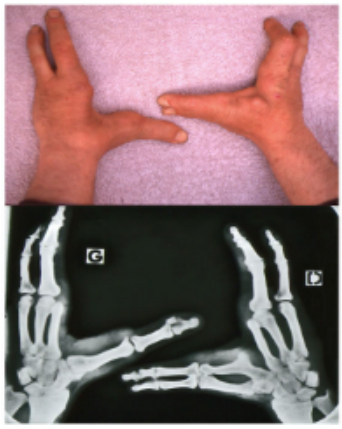


Figure 2.9 Top left to right: the coloboma of II:2; the retina of II:4 exhibiting macular dystrophy and; the coloboma of III:1. The images to the right show the digit phenotype of III:1.

Bilateral colobomata have been associated with other forms of inherited retinal diseases, such as RP (Parmeggiani et al., 2004) and LCA phenotypes (Ozgül et al., 2006). Another association of ocular and digit phenotypes has been seen in Sorsby syndrome, a rare, dominantly inherited combination of bilateral colobomas and apical dystrophy of the hands and feet in the form of brachydactyly type B (Thompson and Baraitser, 1988). A mutation in *ROR2* (receptor tyrosine kinase-like orphan receptor 2) has been found to cause brachydactyly type B but it does not cause Sorsby syndrome (Bacchelli et al., 2003). In 2002, Giltay et al. reported on a patient with iris coloboma in the left eye, choroidal coloboma in the right eye and SHFM and showed this did not result from a mutation in *TP63* (tumour protein p63, mutations in

which cause SHFM4). Another rare condition combining abnormal ocular and digit phenotypes is ectodermal dysplasia, ectrodactyly and macular dystrophy (EEM). This is thought to be an autosomal recessive disorder in which the ocular findings show extensive retinochoroidal atrophy with diffuse retinal pigmentation (Yildirim et al., 2006). Mutations in *CDH3* (cadherin 3) have been found to cause EEM (Kjaer et al., 2005) and hypotrichosis with juvenile macular dystrophy (HJMD; Indelman et al., 2007).

Split-hand/split-foot malformations (SHFM, also called ectrodactyly) are commonly an autosomal dominant trait with reduced penetrance and clinical variability. SHFM is a limb deformity affecting the hands and feet and can present with syndactyly, median clefts and aplasia/hypoplasia of the phalanges, metacarpals and metatarsals. The severity of the phenotype varies within single families and ranges from non-penetrance to syndactyly in mildly affected individuals and a "lobster claw" appearance in severely affected individuals. SHFM is genetically heterogeneous with five SHFM loci identified so far. SHFM1 was linked to 7q21.3 (Crackower et al., 1996), SHFM2 to Xq26 (Faiyaz-Ul-Haque et al., 2005), SHFM3 to 10q24 (Ozen et al., 1999), SHFM4 to 3q27 (Ianakiev et al., 2000) and SHFM5 to 2q31 (Bijlsma et al., 2005). Another family has been linked to 8q21.11-q22.3 (Gurnett et al., 2006). To date, only SHFM4 has a gene identified carrying a causative mutation: *TP63* (Ianakiev et al., 2000). *TP63* (or *p63*) is a homologue of the cell-cycle regulator *TP53*, which plays a critical role in regulation of the formation and differentiation of the apical ectodermal ridge (AER), a critical signalling centre that directs outgrowth and patterning of the developing limb (Saunders, 1948).

DNA rearrangements resulting in copy number variations have been shown to cause SHFM3, which appears to be caused by over-expression of *BTRC* (beta-transducin repeat containing) and suppressor of fused homolog (*Drosophila*), *SUFU* (Lyle et al., 2006). Kano et al. (2005) suggested genome rearrangements are a rare cause of SHFM and found duplications of *LBX1, BTRC, POLL, DPCD* and a disrupted extra copy of dactylin in SHFM subjects. In mice dactylaplasia (*Dac*) is a semi-dominant trait that phenotypically resembles SHFM and mutant alleles of the mouse *Dac* gene cause the disorder (Chai, 1981). It may be that a similar situation is occurring in the SHFM with associated NCMD disorder whereby an increase or decease in copy number is leading to over or under expression of an important gene. Previous genetic analysis will be considered in Chapter 9.

2.5.6 Bull's-Eye Macular Dystrophy

Michaelides et al. (2003b) reported and characterised bull's-eye macular dystrophy, which they mapped to 4p15.2-p16.3. Though the term 'bull's-eye maculopathy' was first used in the description of chloroquine retinopathy (Kearns and Hollenhorst, 1966), bull's-eye lesions have also been reported as features of cone-rod dystrophy and other macular dystrophies (Kurz-Levin et al., 2002). MCDR2 is an autosomal dominant form of bull's-eye maculopathy in which sufferers typically show annular RPE atrophy and central sparing has been suggested to correspond to lipofuscin accumulations in the RPE. The family characterised by Michaelides et al. (2003b) displayed concentric areas of increased AF in the macula (Figure 2.10b) prior to showing ophthalmoscopic evidence of retinal atrophy.

As with the other macular dystrophies being investigated, this disease shows an early age of onset with sufferers exhibiting a

red-speckled macular appearance that later develops into the typical bull's-eye maculopathy. In most individuals the disease is confined to the macular region but electrophysiological evidence in older individuals suggests the disease causes more widespread retinal dysfunction in later stages.

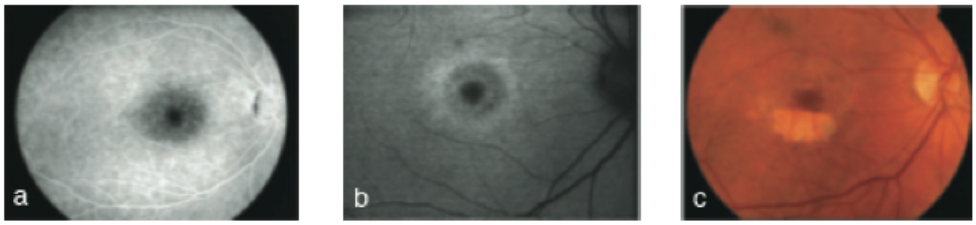


Figure 2.10 Images taken from Michaelides et al. (2003b): a) fluorescin angiography from an affected individual showing localized masking of the choroidal fluorescence in the perifoveal area; b) fundus autofluorescence of the same individual as in a) showing bull's-eye type lesion comprising of decreased perifoveal autofluorescence bordered peripherally and centrally by increased autofluorescnece and c) fundus photograph of another affected individual showing bull's-eye maculopathy with a well-demarcated are of RPE and temporal optic disc pallor.

A mutation in human prominin (mouse)-like-1 has been identified as causing MCDR2 and this will be detailed in Chapter 10.

2.5.7 Bornholm Eye Disease

The final disorder being investigated in this project is the cone dysfunction Bornholm eye disease (BED). This is an early-onset, non-progressive, X-linked disorder in which affected individuals exhibit cone dysfunction and a range of ophthalmologic features, including: myopia, astigmatism, optic nerve head hypoplasia, thinning of the RPE and visible choroidal vasculature. The associated myopia is a severe form and was linked to Xq28 and designated the MYP1 locus. Patients have an abnormal cone ERG, reflecting the cone dysfunction aspect of the disorder. A unique feature of this cone dysfunction compared to others is that all

affected individuals are dichromats and families have been described exhibiting protanopia (Young et al., 2004; Michaelides et al., 2005) and deuteranopia (Haim et al., 1988).

Haim et al. (1988) first described this disorder in a study of a large Danish family from the island of Bornholm with X-linked infantile myopia that also showed astigmatism, impaired vision, hypoplasia of the optic nerve heads and deuteranopia. Schwartz et al. (1990) confirmed linkage to Xq28 and named the disorder Bornholm eye disease and added the clinical signs of ambylopia, reduced ERG flicker function and non-specific pigment abnormalities to the diagnosis. Young et al. (2004) reported on a second family exhibiting the same range of clinical signs. This family were of Danish descent but rather than being deuteranopic, all affected males were protanopic; this family also linked to Xq27-q28. Michaelides et al. (2005) then reported on four British families with an X-linked cone dysfunction associated with myopia and protanopia. As the L and M cone opsin genes are present in a tandem array at the Xq28 linked region, this group conducted molecular analysis on the L/M arrays of these families. Two families were found to have a hybrid L/M gene, which is a common cause of dichromacy but would not explain the cone dysfunction. A third family had the exon 4 mutation Cys203Arg, which is known to cause cone dysfunction if present in one of the first two genes in the array (Winderickx et al, 1992c, Section 1.2.4). The Cys203Arg mutation abolishes opsin function and results in early degeneration of photoreceptors with loss of cone function and colour deficiency (Carroll et al., 2009). The possibility of variations in the L or M opsin genes as the cause of BED was therefore pursued. Further considerations of this are contained in Chapter 11.

2.6 Aims and Objectives

For all the macular dystrophies except MCDR2, the aim of this project is to determine the genetic basis for disease. For this purpose, it was decided that the Affymetrix SNP chips would be used to examine haplotypes and copy number variation. Given that all the disorders except one have been previously linked, this new method was favoured over traditional linkage analysis as it has the potential of providing new insights into the nature of the disease mutation (see Section 4.1). The data obtained should provide a refinement of the disease regions and guide us to the primary candidate genes.

For MCDR2, the aim is to identify new families with *PROM1* mutations with additional individuals exhibiting the bull's-eye phenotype also to be screened. For families in which a mutation is identified, their allele for the marker D4S1601 will be determined for comparison to previously linked families with *PROM1* mutations.

For Bornholm eye disease, two factors indicate the involvement of the X-linked opsin genes: the mapping of the disease locus to Xq28 (the location of the M and L opsin gene array) and the presence of dichromacy in all individuals with this disorder. The aim therefore is to determine whether sequence and/or organisation changes in the L and M opsin genes in affected families are responsible for the disorder.

3. MATERIALS & METHODS

Reagents Used and Methods Applied

3.1 Polymerase Chain Reaction Protocols

3.1.1 BIOTaq

Reactions were conducted using BIOTaq DNA Polymerase (Bioline, BIO-21040) and contained 1µl genomic DNA (50-100ng, or to a specific concentration as stated in chapters). 1µl of each of the forward and reverse primers were added from stock solutions of 10µM, with 1µl dNTP mix (10mM stock each), 2.5µl NH_4 (10x stock), 1.5µl $MgCl_2$ (50mM stock), 0.5µl BIOTaq DNA polymerase (5U/µl stock) and made up to a total 25µl reaction volume with molecular biology grade H_2O. Amplifications were conducted in a Techne TC-412 PCR machine using the standard cycles of an initial denaturation at 95°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 45 seconds, primer annealing for 45 seconds was conducted at specific temperatures for each primer set (given in the disease chapters), followed by a 1 minute extension at 72°C. A final extension at 72°C for 7 minutes was conducted. Any variations to these cycles are stated in the disease chapters.

Gradient PCRs

For the majority of primer sets, initial gradient PCRs were conducted in a Techne Touchgene Gradient PCR machine to determine the optimum annealing temperature for each primer set. BIOTaq or GoTaq (Section 3.1.2) protocols with control genomic DNA were conducted across ten different annealing temperatures: 55.4°C, 56.1°C, 57.2°C, 58.6°C, 59.5°C, 60.3°C, 61.3°C, 62.6°C, 63.9°C and 64.6°C. Figure 3.1 highlights the

usefulness of this method and shows the increase of amplicon production with an increase in temperature and the reduction of production of unwanted amplicons from non-specific primer binding.

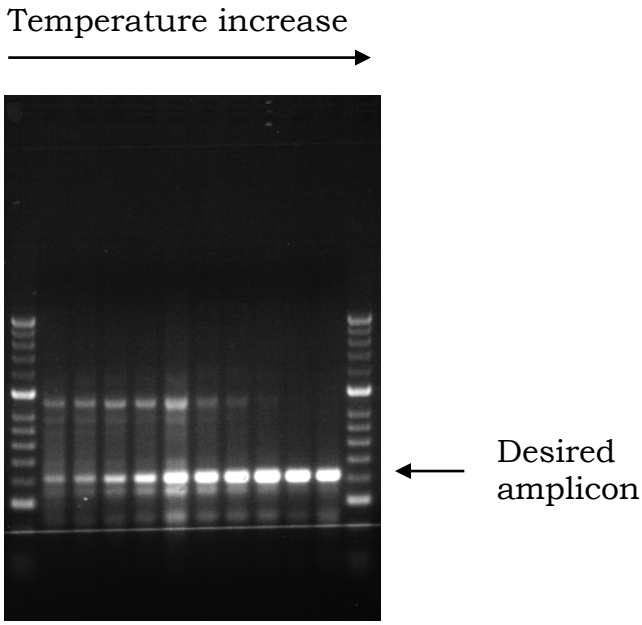


Figure 3.1 Gel image showing PCR products run on a 1% gel for gradient PCRs using varying annealing temperatures listed in the text.

Gel Electrophoresis

Amplicon size determined the percentage of agarose (Sigma-Aldrich, normal: A9539; low-melt: A9414) used for electrophoresis gels. The agarose was dissolved in 2M Tris-acetate, 100mM Na_2EDTA (ethylenediamine tetra-acetic acid) buffer (TAE buffer, 50x stock diluted to 1x; National Diagnostics, EC-872). Fragments <500bp were run on a 2% gel; 500–1,000bp fragments run on a 1% gel and amplicons >1,000bp were run on a 0.5% gel. For very small fragments (<500bp), low-melt agarose gels were used. Gels contained 1µl ethidium bromide (10mg/ml) for every 20mls of gel (final concentration of 500ng/ml). Gels were viewed on a BioDoc-It UV Transilluminator.

Gel Extraction

GoTaq Green reactions always required this form of PCR cleanup due to the green buffer and this protocol was used for cleanup of all PCR products prior to sequencing unless otherwise stated. The desired amplicons were excised from the agarose gel and the DNA extracted using the QIAquick Gel Extraction Kit (QIAGEN, 28706) with the extraction protocol for a microcentrifuge followed. This involved adding three gel volumes of buffer QG (solubilisation and binding buffer; QIAGEN, 19063) per one volume of gel (100mg of gel was given as equivalent to 100µl of buffer). The gel slices were incubated in the buffer at 50°C for 10-15 minutes or until the gel had dissolved. One gel volume of isopropanol was then added and the mixtures vortexed before being transferred to QIAquick spin column. The samples were centrifuged for 1 minute, 13,000rpm and the flow-through discarded. 0.5ml Buffer QG was then added to each column and centrifuged for 1 minute, 13,000rpm. The flow-through was again discarded and 0.75ml Buffer PE (wash buffer; QIAGEN, 19065) added to the columns and left to stand for 5 minutes before being centrifuged for 1 minute, 13,000rpm. The flow-through was discarded and the empty column spun for a further minute at 13,000rpm. The column was then placed in a clean tube and the DNA eluted in 30µl EB Buffer (10mM Tris-Cl, pH 8.5) by being left to stand for 1 minute in the buffer before a final spin at 13,000rpm for 1 minute. The purified DNA was then used in sequencing reactions or for cloning into plasmids (Section 3.3.1).

Montage Cleanup of PCR Products

For PCRs not conducted with GoTaq Green, reactions that produced only a single amplicon could be purified with Montage Mutliscreen PCR µ96 filter plates (Millipore, LSKMPCR10). Each PCR was made up to a volume of 100µl with molecular biology

grade H_2O and the total volume transferred to the purification plate (one reaction per well). A vacuum was applied to the plate (600mbar) for 10 minutes or until the wells were dry and then a further 25µl of molecular biology grade H_2O was added to the wells and the vacuum applied for 3 minutes (or until wells were dry). The vacuum was removed and the underside of the plate blotted dry. 20µl of molecular biology grade H_2O was added to each well and the plate was covered and shaken for 10 minutes using a vortex machine at 1,000rpm. The remaining volume was then transferred from the plate for storage at 4 - $^{-}$20^{o}C.

Sequencing

DNA was added at different volumes depending on the PCR cleanup procedure used. If directly sequenced from PCR products then 3µl were used, 4µl from gel extracted products and 3µl from Montage purified products and purified plasmid. Sequencing was conducted using the Big Dye Terminator v3.1 Cycle Sequencing Kit (ABI, 4337456). 1µl of the forward or reverse primer was added to the DNA (10µM stock) with 0.5µl Big Dye (5x) and 1.5µl Sequencing Buffer. Reactions were made up to 10µl with molecular biology grade H_2O. The reaction cycles included an initial denaturation at 96^{o}C for 1 minute followed by 25 cycles of 96^{o}C for 10 seconds, 50^{o}C for 5 seconds and then 60^{o}C for 4 minutes. Products were then cleaned (below) and analysed in an ABI Prism 3730 DNA Analyser (Applied Biosystems).

Standard Sequencing Cleanup

Following the sequencing reaction, 10µl of molecular biology grade H_2O was added to the 10µl of sequencing products. To this, 0.5µl EDTA (0.5M, pH 8.0) was added with 2µl Sodium Acetate (3M, pH 5.2) and 50µl 100% ethanol. Samples were vortexed, wrapped in foil and incubated at room temperature for 15 minutes. The

samples were then spun at 13,000 rpm for 30 minutes and the eluate carefully removed and discarded. 70µl 70% ethanol was then added and the samples spun at 13,000 rpm for 15 minutes. The eluate was discarded as before and samples dried at 65°C for at least 5 minutes. The samples were then resuspended in 11µl HiDi Formamide (Applied BioSystems, 4440753) prior to analysis in an ABI Prism 3730 DNA Analyser (Applied Biosystems).

Montage Sequencing Cleanup

For this method, the Montage SEQ_{96} Sequencing Reaction Cleanup Kit was used (Millipore, LSKS09604). Following the sequencing reactions, 25µl Injection Solution was added to each sample and the total volume (35µl) was transferred to the cleanup plate. A vacuum (600mbar) was applied to the wells for 3 minutes (or until the wells were dry). A further 25µl Injection Solution was added and the vacuum applied for 4 minutes. The vacuum was removed and the underside of the plate blotted dry. 25µl Injection Solution was then added to each well and the plate covered and shaken on a vortex at 1,000rpm for 10 minutes. The remaining volume was transferred to a sequencing plate for analysis in an ABI Prism 3730 DNA Analyser (Applied Biosystems).

3.1.2 GoTaq

This protocol used GoTaq Green DNA Polymerase (Promega, M3175). Reactions contained 1µl genomic DNA (50-100ng, or a specific concentration as stated in chapters). 1µl of each of the forward and reverse primers were added from stock solutions of 10µM with 1µl dNTP mix (10mM stock of each), 10µl Green Buffer (5x stock), 0.25µl GoTaq DNA polymerase (5U/µl stock) and made up to a 50µl reaction volume with molecular biology grade H_2O. Amplifications were conducted using the same cycles as for the BIOTaq Protocol (Section 3.1.1). Any variations to these cycles are

stated in the disease chapters. Appropriate agarose gels were prepared for electrophoresis as described in Section 3.1.1. PCR products were always gel extracted prior to sequencing.

3.1.3 KOD Hot Start

Reactions were conducted using the KOD Hot Start Polymerase (MERCK, 71086) and contained 1µl genomic DNA diluted (50-100ng or to a specific concentration as stated in chapters). 1.5µl of each of the forward and reverse primers were added from stock solutions of 10µM with 5µl dNTP mix (2mM stock of each), 3µl $MgSO_4$ (25mM stock), 5µl Buffer (10x stock) and 1µl KOD DNA polymerase (1U/µl stock). Reactions were made up to 50µl with molecular biology grade H_2O. PCR cycles consisted of an initial denaturation at 95°C for 2 minutes, followed by 35 cycles of 95°C for 20 seconds and annealing at 62°C (unless otherwise stated in disease chapters) for 10 seconds. The extension was conducted at 70°C with the time varying depending on the amplicon length, for <500bp 10 seconds per kilobase, for 500-1,000bp 15s/kb, for 1,000-3,000bp 20s/kb and for products >3,000bp 25s/kb timings were used. Appropriate agarose gels were prepared for electrophoresis as described in Section 3.1.1 and sequences cleaned prior to analysing.

3.1.4 BIOXACT Long

Reactions were conducted using the BIOXACT Long recommended protocol (Bioline, BIO-21049). 1µl genomic DNA (50-100ng) was used with 5µl OptiBuffer (10x stock), 2.5µl $MgCl_2$ (50mM stock), 1µl dNTP (100mM stock of each), 1µl each of the forward and reverse primers (10µM stock) and 1µl BIOXACT DNA polymerase (4U/µl). Reactions were made up to a 50µl final volume with molecular biology grade H_2O. For amplification of L and M gene exons 3 to 5 (Section 11), the following reagents were used: 5µl

OptiBuffer (10x stock), 1µl $MgCl_2$ (50mM stock), 0.5µl dNTPs (100mM each stock), 2.5µl Hi-Spec Additive, 1µl 50-100ng DNA, 0.3µl of each the forward and reverse primers (100µM stock), 1µl BIOXACT (4U/µl stock) and made up to a 50µl final volume with molecular biology grade H_2O. BIOXACT cycles began with an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of 95°C for 30 seconds, annealing at 62°C for 1 minute with extension at 68°C for 4 minutes and a final extension at 68°C for 10 minutes. Appropriate agarose gels were prepared for electrophoresis as described in Section 3.1.1 and the products cleaned prior to sequencing.

3.1.5 Extensor

Reactions were conducted using the Extensor PCR Master Mix 2 (Thermo Scientific, AB-0793/A). 1µl DNA (50-100ng) was used with 12.5µl Extensor Master Mix (2x stock), 2µl of each of the forward and reverse primers (10µM stock) and made up to 20µl with molecular biology grade H_2O. Cycles involved an initial denaturation at 94°C for 2 minutes followed by 10 cycles of 94°C for 10 seconds, annealing at 64°C (unless otherwise stated in the disease chapters) for 30 seconds and extension at 68°C for 1 minute for products up to 1kb and 2 minutes for products up to 3kb. These cycles were followed by 20 cycles of 94°C for 20 seconds, annealing at 64°C (unless otherwise stated) for 30 seconds and extension at 68°C for 1 or 2 minutes depending on the product length. A final extension at 68°C was run for 7 minutes. Appropriate agarose gels were prepared for electrophoresis as described in Section 3.1.1.

3.1.6 Long Range PCR

This protocol was taken from Oda et al. (2003) and used only for the amplification of first and downstream genes in the L and M X

chromosome array. Reactions were conducted in 0.2ml tubes and prepared on ice. Mixtures were not vortexed but carefully mixed by minimal pipetting. Cyclers were pre-heated. Reactions were carried out using the 5PRIME PCR Extender System (5PRIME, 2900158) and contained 10-50ng genomic DNA, 1.25µl dNTP (10mM stock of each), 1µl of each primer (10µM stock), 2.5µl Tuning Buffer (10x stock), 0.2µl 5PRIME Polymerase (5U/µl stock) and reactions made up to 25µl with molecular biology grade H_2O. The cycling schedules for the first gene reactions involved initial denaturation at 93°C for 3 minutes followed by 10 cycles of 93°C for 15 seconds, 62°C for 30 seconds and 68°C for 15 minutes. This was followed by 18 cycles of 93°C for 15 seconds, 62°C for 30 seconds and 68°C for 15 minutes with 20 second increments for each cycle. For the downstream gene amplifications, cycling was carried out at 93°C for 3 minutes, followed by 10 cycles of 93°C for 15 seconds then 68°C for 15 minutes. These were followed by 18 cycles of 93°C for 15 seconds then 68°C for 15 minutes with 20 second increments per cycle. The large fragments were analysed on a 0.5% agarose gel and the PCR products used in subsequent BIOTaq reactions (see Section 11.4.3).

3.1.7 SYBR Green Quantitative PCR

Triplicate reactions for each sample were conducted using the Power SYBR Green PCR Master Mix (Applied Biosystems, 4367659). Genomic DNA was purified (Section 3.2.1) and 10ng used per single reaction. 0.625µl of each primer (5µM stock) was added to the DNA with 6.25µl Power SYBR Green Master Mix (2x stock) and 4µl molecular biology grade H_2O. The final reaction volume was 12.5µl and transferred to a BIO-RAD Hard-Shell 96 microplate (BIO-RAD, HSA-9601). Cycles were conducted in a BIO-RAD C1000 Thermal Cycler and involved a 95°C initial denaturation for 10 minutes followed by 40 cycles of 95°C for 15

seconds and 62°C for 1 minute. A melt curve of 95°C for 15 seconds and 60°C for 15 seconds was added to the cycles to ensure specificity of primer binding.

3.1.8 TaqMan QPCR

Reactions were conducted following the protocol from Neitz and Neitz (2000) and used to amplify exon 5 of the L and M opsin genes. A single primer set hybridised to both L and M gene sequences and two probes were used, one specific for L and tagged at the 5' end with 6FAM and the other probe specific for M and tagged at the 5' end with JOE. Both probes were tagged at the 3' end with the quencher TAMARA. Primers and probes are listed in Table 11.1, Section 11.4.3. Reactions for each sample were run in triplicate with each single reaction containing 10ng of purified DNA (Section 3.2.1), 0.75µl L probe (4.6µM stock), 1.25µl M probe (5µM stock), 0.9µl of each of the forward and reverse primers (25µM stock) and 12.5µl TaqMan Gene Expression Master Mix (Applied BioSystems, 4369016). Reactions were made up to a final volume of 25µl with molecular biology grade H_2O and transferred to BIO-RAD Hard-Shell 96 microplate (BIO-RAD, HSA-9601). Cycles were conducted in a BIO-RAD C1000 Thermal Cycler and the cycling schedule involved an initial incubation at 50°C for 2 minutes followed by denaturation at 95°C for 10 minutes. This was followed by 3 cycles of 95°C for 30 seconds and 67°C for 1 minute then 27 cycles of 95°C for 15 seconds and 67°C for 1 minute.

3.1.9 Genotyping of D4S1601

BIOTaq reactions were conducted to amplify the marker D4S1601 but using half the normal protocol primer concentration, 10ng of DNA and an annealing temperature of 56°C. The forward primer was labelled at the 5' end with 6FAM. On completion of the PCR

cycles, 0.5µl of the PCR product was added to 0.5µl 500 LIZ Size Standard (Applied BioSystems, 4366589) and 9µl of HiDi Formamide (Applied BioSystems, 4440753). The mixture was heated at 95°C for 5 minutes before placing on ice for 2 minutes. The samples were then analysed in an ABI Prism 3730 DNA Analyser (Applied Biosystems).

3.1.10 Digestion of PCR Product

The protocol published by Ueyama et al. (2003) was attempted to determine the ratio of L to M opsin gene promoters. GoTaq PCRs were conducted to amplify the promoter regions from the same primer set (Table 11.1, Section 11.4.3). The products were gel extracted with 30µl of the eluted product used in the digestion. 5µl of NEB Buffer 4 (10x stock; New England BioLabs, B7004S) was added to the extracted DNA with 1.5µl *Bsr* FI (10U/µl stock; New England BioLabs, R0562S) and 13.5µl molecular biology grade H_2O. The reactions were incubated at 37°C for one hour before adding 2.5µl 10% sodium dodecyl sulphate (SDS) to each sample prior loading on a 3% low-melt agarose gel. Gels were stained with TAE buffer with SYBR Green I 10,000x concentrate in DMSO (dimethyl sulfoxide, 1µl for every 10ml buffer; Invitrogen, S7563). Products from the first gene promoter were expected to give 137bp and 32bp bands and those from the downstream promoters were expected to give 97bp, 40bp and 32bp bands. The ratio of the 137bp and 97bp fragments were intended to be used to determine the ratio of L to M promoters.

3.2 Affymetrix Protocols

3.2.1 DNA Preparation

0.1 volumes of 3M Sodium Acetate were added to stock samples of genomic DNA. 2.5 volumes of 100% ethanol were then added to

each sample and mixtures vortexed and either left to precipitate overnight at -20°C or for one hour at -80°C. Samples were then spun at 4°C, 12,000rpm for 20 minutes. The supernatant was carefully aspirated and discarded. 1ml of cold 70% ethanol was then added and the samples spun at 4°C, 12,000rpm for 10 minutes. The supernatant was again carefully aspirated and samples left to air-dry for an hour. Each sample was then dissolved in 10µl EB buffer. The concentration of each sample was determined using a Nanodrop 2000 Spectrophotometer (Thermo Scientific). For the Affymetrix protocols, 250ng of DNA was required.

3.2.2 50K *Xba* I Protocol

For this method, the GeneChip Human Mapping 50K *Xba* Assay Kit (Affymetrix, 900521) was used. The 100K low-throughput protocol provided by Affymetrix was adhered to with the basic steps included here. The 250ng of purified DNA was added to 2µl Buffer D (10x stock; Promega, R004A), 2µl Bovine Serum Albumin Acetylated (1mg/ml stock; Promega, R396D), 1µl *Xba* I (12U/µl stock; Promega, R618A) and made up to 20µl with molecular biology grade H_2O. The DNA was digested at 37°C for 120 minutes, followed by enzyme inactivation at 70°C for 20 minutes. Adaptors were then ligated to the *Xba* I sticky ends of the DNA in a reaction containing 1.25µl Adaptor *Xba* I (5µM stock; Affymetrix, 900410), 2.5µl T4 DNA ligase buffer with 10mM ATP (10x stock; New England BioLabs, B0202S), 0.625µl T4 DNA ligase (400U/µl stock; New England BioLabs, M0202S), 0.625µl molecular biology grade H_2O and the 20µl of digestion mixture. Ligations were carried out for 120 minutes at 16°C with an enzyme inactivation step of 20 minutes at 70°C. 75µl molecular biology grade H_2O was then added to each ligation sample and 10µl of each diluted ligation used in a PCR reaction, three reactions per sample. The

Platinum *Pfx* DNA Polymerase was used for the PCRs (2.5U/µl stock; Invitrogen, 11708-021). Each single reaction contained: 10µl *Pfx* Buffer (10x stock), 10µl PCR Enhancer (10x stock), 2µl $MgSO_4$ (50mM stock), 12µl dNTPs (2.5mM stock of each), 10µl Primer 001 (Affymetrix, 900409), 2µl Platinum *Pfx* polymerase and 10µl diluted ligation and was made up to 100µl with molecular biology grade H_2O. Mixtures were briefly vortexed and spun at 2,000rpm for one minute. Cycles involved initial denaturation at 94°C for 3 minutes followed by 30 cycles of 94°C for 15 seconds, 60°C for 30 seconds and 68°C for 60 seconds. A final extension at 68°C for 7 minutes was then run. PCR products were analysed on a 2% agarose gel and successful PCR products purified. The three PCR reactions for each single sample were combined for purification into one well of a QIAGEN MinElute 96 UF Plate (QIAGEN, 1019566). A vacuum of 600mbar was applied to the plate and when the wells were dry, 50µl molecular biology grade H_2O was added and the vacuum again applied until the wells were dry. This wash step was repeated twice more. 40µl EB Buffer was then added to each well and the plate sealed and shaken for 5 minutes at room temperature. The eluate from each well was transferred to a fresh tube and the concentration of each sample quantified using a Nanodrop. 40µg DNA was required for the fragmentation process, which was made up to a volume of 45µl in EB buffer. The GeneChip Fragmentation Reagent (2.5U/µl stock; Affymetrix 900131) was diluted to 0.04U/µl in molecular biology grade H_2O and Fragmentation Buffer (10x stock with a final concentration of 1x; Affymetrix, 900422). 5µl of this mixture was then added to the 45µl of purified DNA and fragmentation conducted at 37°C for 35 minutes followed by enzyme inactivation at 95°C for 15 minutes. The fragmentation products were then viewed on a 4% TAE agarose gel. Successful fragmented products were labelled with the GeneChip DNA Labelling Reagent (7.5mM

stock; Affymetrix 900484). Per one reaction, 14µl Terminal deoxynucleotidyl Transferase (TdT) Buffer (5x stock; Affymetrix 901022), 2µl Labelling Reagent and 3.5µl TdT (30U/µl stock; Affymetrix, 901023) were added to the fragmented products. The hybridisation of the labelling reagent was conducted at 37°C for 2 hours followed by enzyme inactivation at 95°C for 15 minutes. Samples were then sent to the UCL Wolfson Institute for hybridisation to the array.

3.2.3 250K *Sty* I Protocol

For this method the GeneChip Mapping 250K *Sty* Assay Kit was used (Affymetrix, 900765). The 500K low-throughput protocol provided by Affymetrix was adhered to but the basic steps are included here. The 250ng of purified DNA was added to 2µl NEB Buffer 3 (10x stock; New England BioLabs, B7003S), 2µl Bovine Serum Albumin Acetylated (1mg/ml stock; Promega, R396D), 1µl *Sty* I (10U/µl stock; New Enlgand BioLabs, R0500S) and made up to 19.75µl with molecular biology grade H_2O. The DNA was digested at 37°C for 120 minutes, followed by enzyme inactivation at 65°C for 20 minutes. Adaptors were ligated to the *Sty* I sticky ends of the DNA in a reaction containing: 0.75µl Adaptor *Sty* I (50µM stock; Affymetrix, 900597), 2.5µl T4 DNA ligase buffer with 10mM ATP (10x stock; New England BioLabs, B0202S), 2µl T4 DNA ligase (400U/µl stock; New England BioLabs, M0202S) and the digestion mixture. Ligations were carried out for 180 minutes at 16°C with an enzyme inactivation step of 20 minutes at 70°C. 75µl molecular biology grade H_2O was then added to each ligation sample and 10µl of each diluted ligation used in a PCR reaction, with three reactions per sample. The Clontech Titanium DNA Amplification Kit was used for the PCRs (Clontech, 639240). Each single reaction contained 10µl Titanium Taq Buffer (10x stock), 20µl G-C Melt (5M stock), 14µl dNTPs (2.5mM stock of each), 4.5µl

Primer 002 (100µM stock; Affymetrix, 900595), 2µl Titanium Taq Polymerase (50x stock) and made up to 100µl with molecular biology grade H_2O. Mixtures were briefly vortexed and spun at 2,000rpm for one minute. Cycles involved initial denaturation at 94°C for 3 minutes followed by 30 cycles of 94°C for 30 seconds, 60°C for 30 seconds and 65°C for 15 seconds. A final extension at 68°C for 7 minutes was then run. PCR products were analysed on a 2% agarose gel and if successful PCRs were evident the products were purified. For purification, 8µl 0.1M EDTA were added to each PCR reaction and samples briefly vortexed and spun. The three PCR reactions for each single sample were combined for purification into one well of a QIAGEN MinElute 96 UF Plate (QIAGEN, 1019566). A vacuum of 600mbar was applied to the plate and when the wells were dry, 50µl molecular biology grade H_2O was added and the vacuum again applied until the wells were dry. This wash step was repeated twice more. 45µl Recovery Buffer (RB; Clontech, 636976) was then added to each well and the plate sealed and shaken for 10 minutes at room temperature. The eluate from each well was transferred to a fresh tube and the concentration of each sample quantified using a Nanodrop. 90µg DNA was required for the fragmentation process, which was made up to a volume of 45µl in RB buffer. The GeneChip Fragmentation Reagent (2.5U/µl stock; Affymetrix 900131) was diluted to 0.05U/µl in molecular biology grade H_2O and Fragmentation Buffer (10x stock with a final concentration of 1x; Affymetrix, 900422). 5µl of this mixture was then added to the 45µl of purified DNA and fragmentation conducted at 37°C for 35 minutes followed by enzyme inactivation at 95°C for 15 minutes. The fragmentation products were then viewed on a 4% TAE agarose gel. Successful fragmented products were labelled with the DNA Labelling Reagent (30mM stock; Affymetrix 900778). Per one reaction, 14µl TdT Buffer (5x stock; Affymetrix 901022), 2µl

Labelling Reagent and 3.5µl TdT (Affymetrix, 901023) were added to the fragmented products. The hybridisation of the labelling reagent was conducted at 37°C for 4 hours followed by enzyme inactivation at 95°C for 15 minutes. Samples were then sent to the UCL Wolfson Institute for hybridisation to the array.

3.3 Cloning

3.3.1 Ligation

The ligation of PCR fragments for cloning were conducted using 5.5µl of gel extracted insert (Section 3.1.1), 0.5µl pGEM-T Easy Vector System (Promega, A1360), 1µl T4 DNA ligase buffer with 10mM ATP (10x stock; New England BioLabs, B0202S), 1µl T4 DNA ligase (400U/µl stock; New England BioLabs, M0202S) and made up to 10µl with molecular biology grade H_2O. Reactions were incubated at 4°C for at least 16 hours.

3.3.2 Transformation

JM109 Competent Cells (>10^8cfu/µg; Promega, L2001) were thawed on ice and used for cloning pGEM-T vectors. 25µl aliquots were prepared and 5µl of the pGEM-T ligation mixture added to the competent cells by gentle pipetting and swirling. Cells were kept on ice for 30 minutes before being heat-shocked at 42°C for 1 minute and 30 seconds. Cells were then placed back on ice for 2 minutes. 200µl Super Optimal broth with Catabolite repression (S.O.C) medium (Invitrogen, 15544-034) was added to each tube of cells and mixed by pipetting. The mixture was transferred to a 15ml Falcon tube and incubated in a shaker at 37°C for 1 hour. The cells were then poured and spread on agar plates containing 1.5% agar (Sigma-Aldrich, A1296), 1% tryptone (Sigma-Aldrich, T7293), 0.5% yeast extract (Sigma-Aldrich, Y1625), 1% NaCl, ampicillin (sodium salt, final concentration 100µg/ml;

Calbiochem, 171254), 5-bromo-4-chloro-3-indolyl-beta-D-galactoside (X-Gal, final concentration of 80µg/ml; Calbiochem, 71077) and isopropyl β-D-1-thiogalactopyranoside dioxane-free, high purity (IPTG, final concentration of 0.5mM; Calbiochem, 420322) and left to dry for 30 minutes. Plates were then incubated at 37°C for 16 hours and isolated colonies selected via the blue/white screening method (with white colonies containing the insert). Cells from each single colony were incubated in 4ml of Luria broth (LB; Sigma-Aldrich, L3522) with ampicillin (100µg/ml final concentration) at 37°C in a shaker for 16 hours. Following incubation the cells were spun for 10 minutes, 3,500rpm at 4°C. The supernatant was discarded and the pellet frozen prior to extracting the plasmids.

3.3.3 Plasmid Extraction

Plasmids were extracted from the collected JM109 cells using the GenElute Plasmid Miniprep Kit (Sigma-Aldrich, PLN350). The protocol provided with the kit was followed with cells resuspended in 200µl Resuspension Solution then transferred to a 1.5ml tube. 200µl of Lysis Solution was added to each resuspension and the contents mixed by inversion of the tubes. 350µl Neutralisation/Binding Solution was then added and again mixed by inversion of the tubes, which were then spun at 13,000rpm for 10 minutes. The GenElute Binding Columns were prepared and the clear lysates transferred to the columns, which were spun at 13,000rpm for 1 minute. The flow-through was discarded and 500µl Optional Wash Buffer applied to each column and spun at 13,000rpm for 1 minute and the flow-through discarded. 750µl Wash Solution was then added to each column and spun at 13,000rpm for 1 minute and the flow-through discarded. The empty columns were spun once more for 2 minutes before being transferred to a fresh tube. 50µl of Elution Solution was then

added to each column and spun at 13,000rpm for 1 minute. Plasmids could then be used in sequencing reactions (Section 3.1.1).

4. COMPUTATIONAL METHODS

4.1 Linkage Analysis

To find the causes of inherited disorders, various methods can be used. For autosomal dominant disorders linkage analysis using markers can be conducted to identify the disease locus within the genome, whereas for more complex disorders such as AMD, twin, population and association studies are required. Twin studies allow for genetic and environmental factors important to the disease state to be investigated, as do population studies, and combined they provide detailed information on contributory factors of complex disorders (AMD Section 2.3). This project investigates autosomal dominant disorders for which traditional linkage analysis has already been conducted and located large disease regions. Linkage analysis has long been used to identify the region of the genome in which the genetic fault causing the disease state lies. Following identification of the disease region, candidate genes can be selected and their coding exons screened for mutations. Linkage analysis involves using DNA markers, such as restriction fragment length polymorphisms (RFLPs), microsatellite markers or SNPs, to establish a link between them and the disease locus. RFLP markers involve amplifying specific fragments of the genome and then digesting the amplicon with a particular restriction enzyme. The resulting products of the digest identify the marker haplotype by the combination of digest fragments produced, which is generally represented as a number. For microsatellite markers, the region surrounding the marker is used to design PCR primers, which amplify the marker and the allele is scored by product length. The different fragments are separated and analysed using GeneMapper Software (Applied Biosystems), which provides the haplotype identification based on

the fragment length. The more variations possible for a marker the more informative it can be in linkage analysis.

When a family with an autosomal dominant disorder is identified, initial linkage analysis needs to use markers from over the entire genome in order to find those that link to the disease. This analysis obtains a LOD score, which represents the chance of a particular marker being linked to the disease locus. The LOD score is a logarithm (base 10) of odds score and compares the likelihood of two loci being linked to the likelihood of observing such a thing by chance. For this reason, a LOD score of 3.0 or greater is deemed to represent significant linkage as it shows the odds are 1,000:1 that the linkage observed did not occur by chance.

Linkage analysis depends on the occurrence of recombination events. Recombination can occur between two strands of DNA during meiosis in which the strands align before being separated into different cells. When similar DNA sequences align there is a chance the maternal and paternal strands may switch equivalent sections of DNA. Alleles are defined as unlinked when they display 50% recombination, as described by Mendel's law of independent assortment. The recombination frequency (RF, θ), is the frequency at which a single crossover will occur between two genes during meiosis. If there is a 1% RF (i.e. 1 in 100 chance of recombination), this represents genes being 1 centimorgan (cM) apart. Therefore, when two genes are close together they do not assort independently and are linked.

For the disorders MCDR1, MCDR2, MCDR3, MCDR4 and PBCRA, previous linkage analysis has been conducted using microsatellite markers across all chromosomes to link the disorders to

particular areas of the genome (loci). For MCDR1, this has been done by many research groups (Section 5) and was recently refined by Yang et al. (2007). But for all five macular disorders the regions contain numerous genes as possible candidates, particularly MCDR3 and MCDR4. As the disorders have previously been linked, it is unlikely that study of the same families with microsatellites will refine the disease loci. Single nucleotide polymorphisms (SNPs) are a relatively new form of marker and occur much more frequently over loci than do microsatellites. However, they are not as informative as microsatellites as only two alleles generally exist. But, given their frequency in the genome, SNP analysis will be used in this project to see if their frequency across the loci will provide informative data that will help refine the previously linked disease regions.

4.2 Use of the Affymetrix Genotyping Console

The Affymetrix data can be used in two ways: the first makes use of the data that provide genotype calls at approximately 250,000 or 50,000 SNPs (depending on the chip) throughout the genome. By comparing the genotypes of affected individuals at the disease loci, it should be possible to determine which alleles are common to affecteds in the disease region. A limitation of the Affymetrix method of analysis is that the Genotyping Console software cannot determine the phase of alleles. If allele A is present it provides the output AA and if alleles B is present it provides the output BB and when both are detected it provides the output AB. However, it cannot tell which alleles are on which chromosome. This means that in order to determine haplotype either unaffected family members or parent-offspring pairs need to be analysed. In this project affected parent-offspring will generally be analysed and their genotypes compared to other family members. If within

the disease region there are SNPs at which the affected individuals contain a different allele then these SNPs can be excluded as it would be expected that affected individuals would have similar alleles segregating with the disease. For example, if at a particular SNP four samples have the genotype AA and two the genotype BB then an "Exclude" output can be given because amongst the affected individuals there is not a consistent allele. However, if consistent differences are seen this may be evidence of different haplotypes. If excludes can be made this will refine the disease regions and hopefully highlight candidate genes.

The second use of the Affymetrix technology is that it can identify copy number variations (CNV) in the sample genomes. This is important as gene duplications and/or deletions may be associated with the disease states and the gene chips allow identification of microdeletions/duplications. For this analysis, data from affected individuals are compared to data from the International HapMap Project (International HapMap Consortium, 2003). This collection represents "normal" sample data for individuals of selected ethnic origin and is used as reference data for the CNV calculations that determine copy number state changes in the genomes of test samples. Some sites of the genome are known to vary in copy number (Redon et al., 2006; Kidd et al., 2008; Itsara et al., 2009) and the Affymetrix Genotyping Console detects when a sample CNV overlaps with a known variation site. The Genotyping Console integrates information from the database of genomic variants (DGV), which is also available on Ensembl so any CNVs identified can be compared at these sites to identify any that are unique to the affected samples.

A switch was made from the 250K to the 50K GeneChips during this project because it was evident after using the 250K chips that

many of the SNPs were uninformative due to apparent interference from neighbouring SNPs, resulting in numerous NoCall outputs at a 0.1 confidence, a NoCall indicating the console cannot call the genotype under the given parameters (Section 4.3).

Affymetrix supplies detailed explanations of the methods of their calculations and analysis (www.affymetrix.com). The process of the analysis conducted in this project is outlined below.

4.3 Genotype Analysis

Hybridisation of the sample DNA to the chip was carried out by the UCL Wolfson Institute and the chip signals were translated into intensity data that could then be analysed in the Affymetrix Genotyping Console. The chip data are first input through various quality control (QC) calculations to determine the general quality of the data, the output of which reflects how useful the data will be.

4.3.1 Genotype Calls

A QC Call Rate is generated that estimates the overall quality of a sample based on the BRLMM algorithm. The overall call rate for each sample is given and derived from the individual call rate of each SNP across the chip. Affymetrix state that an overall call rate of >=93% is a good indication that the chip data will be useful. However, the correlation between the QC call rate and genotyping performance is not perfect and it is important to remember that a sample may pass the QC yet still have a sub-optimal genotyping performance. Other checks to be made at this point include the confirmation of the console-determined gender of each sample with the actual gender. This gender analysis provides gender calls

estimated from the number of heterozygous calls (the percentage of AB genotype calls) for SNPs on the X chromosome.

The confidence of a call is more important than the call itself as it reflects how certain the Genotyping Console is that the genotype (call) given is the actual genotype at a particular SNP. The confidence is the maximum score at which the algorithm will make a genotype call. The lower the confidence value the more trustworthy the data because the chance of the determined genotype being a miscall is reduced. Calls with a confidence value greater than the user-defined threshold are given a NoCall output. The analysis on the 250K chips had to be run at a confidence of both 0.5 and 0.1, which is not ideal. It was found that by reducing the confidence to 0.1 the number of informative SNP calls was reduced by nearly 50% for many samples. In Figure 4.1a, the difference in the number of NoCalls given at a confidence of 0.1 compared to 0.5 at the same SNPs is evident. It is for this reason that the subsequent batch of samples was run on 50K *Xba* I GeneChips. Though there are five times fewer SNPs, the data were very reliable with over 90% of calls made at a confidence level of 0.1 (Figure 4.1b).

The console genotyping analysis produces a large amount of data for each SNP that must be exported chromosome by chromosome to Microsoft Excel under specific parameters before different samples can be aligned. By doing this, the genotypes at each specific SNP on a chromosome and within a linked disease region can be compared and analysed using basic excel equations.

a)

					CONFIDENCE 0.5					CONFIDENCE 0.1				
Chromosome	Probe Set ID	dbSNP RS ID	Physical Position	Cytoband	Patient I	Patient II	Patient III	Patient IV	Patient V	Patient I	Patient II	Patient III	Patient IV	Patient V
5	SNP_A-4250692	rs16869936	2604254	p15.33	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA
5	SNP_A-1852426	rs9313003	2614928	p15.33	BB	AB	AB	BB	NoCall	NoCall	NoCall	AB	BB	NoCall
5	SNP_A-4288317	rs424339	2620168	p15.33	AA	AB	AB	AA	AA	AA	NoCall	NoCall	AA	AA
5	SNP_A-4285699	rs16870028	2622711	p15.33	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA
5	SNP_A-1980313	rs404016	2628174	p15.33	BB	AB	AB	BB	BB	BB	AB	AB	NoCall	BB
5	SNP_A-1980314	rs434958	2628351	p15.33	BB	AB	AB	BB	BB	BB	AB	AB	BB	BB
5	SNP_A-1980315	rs380259	2628392	p15.33	AA	AB	AB	AA	AA	AA	AB	AB	NoCall	AA
5	SNP_A-4285155	rs435098	2633144	p15.33	AB	AA	AA	AA	NoCall	AB	NoCall	NoCall	NoCall	NoCall
5	SNP_A-2299176	rs6893795	2637026	p15.33	BB	AB	AB	AB	BB	BB	AB	AB	AB	BB
5	SNP_A-4257233	rs225752	2641264	p15.33	BB	BB	BB	BB	AB	BB	BB	BB	NoCall	NoCall
5	SNP_A-2123628	rs7726088	2641400	p15.33	BB	BB	BB	BB	BB	BB	NoCall	BB	BB	BB
5	SNP_A-1980316	rs7702236	2656309	p15.33	AA	AA	AA	AA	AA	NoCall	AA	NoCall	NoCall	NoCall
5	SNP_A-1980318	rs2004187	2665747	p15.33	AB	AB	AB	BB	BB	AB	AB	AB	BB	BB
5	SNP_A-2212432	rs9313007	2668290	p15.33	AA	AA	AA	AA	AA	AA	NoCall	AA	AA	AA
5	SNP_A-2163469	rs2441942	2669477	p15.33	BB	AB	AB	BB	BB	BB	AB	AB	BB	BB
5	SNP_A-4248476	rs16870184	2692096	p15.33	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB
5	SNP_A-2207253	rs16870220	2697862	p15.33	BB	BB	BB	BB	BB	NoCall	NoCall	BB	NoCall	BB
5	SNP_A-2309515	rs323660	2698414	p15.33	AB	BB	BB	AB	BB	AB	BB	NoCall	AB	NoCall
5	SNP_A-2225667	rs17608672	2698565	p15.33	BB	BB	BB	BB	AB	NoCall	BB	NoCall	NoCall	NoCall
5	SNP_A-2180839	rs1393235	2702046	p15.33	AA	AA	AA	AA	AA	AA	AA	NoCall	AA	AA
5	SNP_A-4284686	rs2962558	2717086	p15.33	AB	BB	BB	BB	BB	AB	BB	BB	BB	BB
5	SNP_A-1980322	rs2937761	2717245	p15.33	AB	AB	AB	AB	AB	AB	AB	AB	AB	AB
5	SNP_A-1873076	rs950865	2720684	p15.33	BB	BB	BB	BB	BB	BB	BB	BB	NoCall	BB
5	SNP_A-2096741	rs10042330	2721187	p15.33	AB	AB	AB	AB	AB	AB	AB	AB	AB	AB
5	SNP_A-1942558	rs10054312	2727526	p15.33	AB	AB	AB	AB	BB	AB	AB	AB	AB	BB
5	SNP_A-1935629	rs7704762	2732511	p15.33	AA	AA	AB	AA	AA	NoCall	AA	AB	NoCall	AA
5	SNP_A-1855408	rs2935620	2738567	p15.33	AB	AB	AA	AB	AA	AB	AB	AA	NoCall	AA
5	SNP_A-2142788	rs2935621	2738812	p15.33	AB	AB	AA	AB	AA	NoCall	AB	AA	AB	AA
5	SNP_A-1810773	rs2935624	2741597	p15.33	AB	AB	AA	AA	AA	AB	AB	AA	AA	AA
5	SNP_A-2241061	rs16870285	2741941	p15.33	NoCall	BB	BB	BB	BB	NoCall	NoCall	BB	BB	NoCall
5	SNP_A-1789827	rs16870313	2749844	p15.33	BB	BB	BB	BB	BB	NoCall	NoCall	NoCall	NoCall	NoCall
5	SNP_A-4252543	rs904124	2750960	p15.33	BB	BB	AB	AB	AB	BB	BB	AB	AB	AB

b)

					CONFIDENCE 0.1			
Chromosome	Probe Set ID	dbSNP RS ID	Physical Position	Cytoband	Patient I	Patient VI	Patient II	Patient III
5	SNP_A-1669859	rs1827192	1674184	p15.33	AA	AA	AA	AA
5	SNP_A-1682530	rs1968011	1677351	p15.33	AB	AA	AB	AB
5	SNP_A-1652172	rs6869105	1820250	p15.33	AA	AB	AB	AA
5	SNP_A-1675736	rs6555016	1892458	p15.33	BB	AB	AB	BB
5	SNP_A-1751523	rs10512646	1892498	p15.33	AB	BB	BB	BB
5	SNP_A-1669617	rs10512638	2030575	p15.33	BB	BB	BB	BB
5	SNP_A-1675465	rs4129462	2159236	p15.33	BB	BB	BB	BB
5	SNP_A-1737281	rs1379917	2284570	p15.33	AA	NoCall	AA	AA
5	SNP_A-1734628	rs117622	2373003	p15.33	AB	AA	AB	AB
5	SNP_A-1749439	rs10512685	2484374	p15.33	AA	AA	AA	AA
5	SNP_A-1715289	rs404016	2628174	p15.33	BB	AA	AB	AB
5	SNP_A-1668835	rs415721	2628943	p15.33	AA	BB	AB	AB
5	SNP_A-1749809	rs1374568	2657660	p15.33	AA	BB	AB	AB
5	SNP_A-1723712	rs323685	2657686	p15.33	AB	BB	AB	AB
5	SNP_A-1664236	rs10512678	2657903	p15.33	AA	BB	AB	AB
5	SNP_A-1648732	rs10512677	2664996	p15.33	AA	AA	AA	AA
5	SNP_A-1697240	rs10512672	2855065	p15.33	AA	AB	AB	AA
5	SNP_A-1694777	rs438418	2902436	p15.33	AA	AA	AA	AA
5	SNP_A-1706977	rs9313016	2972588	p15.33	BB	NoCall	BB	BB
5	SNP_A-1651722	rs314014	3116045	p15.33	AA	AA	AA	AA
5	SNP_A-1741057	rs463758	3201532	p15.33	BB	AB	BB	AB
5	SNP_A-1737605	rs1661121	3203829	p15.33	BB	BB	BB	BB
5	SNP_A-1672264	rs4131703	3355116	p15.33	BB	NoCall	AB	AB
5	SNP_A-1738304	rs10512699	3490548	p15.33	BB	AA	AB	AB
5	SNP_A-1652464	rs958909	3582485	p15.33	AA	AB	AA	AB
5	SNP_A-1689713	rs4866602	3742363	p15.33	AB	AA	AB	AB
5	SNP_A-1741939	rs1502641	3752935	p15.33	AA	AB	AB	AA
5	SNP_A-1663866	rs979132	3826651	p15.33	AB	BB	BB	BB
5	SNP_A-1695909	rs2008927	4007570	p15.33	AB	BB	BB	BB
5	SNP_A-1755342	rs492724	4084526	p15.33	AB	BB	BB	BB
5	SNP_A-1707697	rs1187468	4118697	p15.33	BB	AB	AB	BB
5	SNP_A-1754166	rs1187474	4146237	p15.33	BB	AB	AB	BB

NoCall

Figure 4.1 Excel images highlighting the NoCalls (blue) received from the Affymetrix Genotyping Console: a) the increase in NoCalls received from the 250K microarrays when changing from a confidence of 0.5 to 0.1 is evident and b) shows some data received at 0.1 confidence using the 50K microarrays.

4.3.2 Trio Data

Mendel Check

Where trio data are available (affected and unaffected parents plus affected offspring) the samples are checked for Mendelian

inheritance i.e. to ensure that the offspring has inherited one allele from each parent. A calculation for this was developed as an Excel Macro by Dr. Andrew Webster, which looks at a row of genotypes and assumes the first two are the parent genotypes and those following are from the offspring. It then tests each offspring genotype to ensure each of the alleles is inherited from a separate parent. The outputs given are either MenOK, indicating the genotypes are consistent with Mendelian inheritance, or Error_N, indicating an error in N (number of) offspring (Figure 4.2). Any errors in the Mendel check are likely to be miscalls but may also arise from hemizygosity of one allele, resulting in an apparent homozygous call. For example, if the affected parent is AA, the unaffected parent is BB but the single affected offspring is AA the Mendel check will produce an Error_1 output. The Genotyping Console does not differentiate how many alleles are present. If it detects alleles A and B, it will provide the ouput AB but if B is not detected the output will be given as AA whether there are two A alleles or not. This means that runs of apparent homozygous calls may be representative of a deletion and the Mendel check allows for identification of these regions. Typically a single error will be detected on one chromosome and the likely cause will be a miscall, hence it is important to use calls at a confidence of 0.1. Alternatively, it may be indicative of the offspring losing the allele from one parent (resulting in hemizygosity) and deletions may therefore be identified from this analysis.

Final Call

With trio data, the dominantly inherited allele associated with the disorder can be deduced by a second Macro equation, ADTriad (Figure 4.2). This uses information from the unaffected parent to assign the allele of the affected parent inherited by the offspring, such as in a case where the affected child and the affected parent

are both AB but the unaffected parent is BB. From this it can be determined that the SNP inherited with the dominant disease is A. In this example, the output for the affected child is listed under 'Paternal Allele' and given as AA but it is important to remember this does not represent the SNP alleles but rather the allele inherited from the affected parent. The Error_N calls from the Mendel check can then be corrected to AB (if they are isolated and therefore likely to be a miscall) and combined with the column of 'Paternal Alleles' to give the 'Final Call' of the affected offspring (Figure 4.2). It is important to do this analysis and run unaffected samples when possible because the Affymetrix chip cannot determine phase, i.e. the order of alleles for each chromosome. Thus the only way to determine which alleles are inherited between affecteds is to compare them to the unaffected parent as described above.

4.3.3 Autosomal Dominant Allele of Affecteds

Following the Mendel check and correction of Final Calls, the genotypes of all affected individuals for a particular disease type can be compared side-by-side and another Macro equation, AD (autosomal dominant), used to determine the allele inherited across each SNP amongst affecteds and to exclude SNPs where the genotypes differ. An Exclude output is given when, for example, four samples from affected individuals have the genotype AA but two are BB. The exclude output is given because amongst affected individuals there is no consistent allele and it is expected for affected individuals to have common SNPs segregating with the disease. For this reason, where differences are encountered it is judged acceptable to exclude regions of DNA where the genotypes of affected individuals differ. If there are consistent differences within a known disease region it may be indicative of different disease haplotypes.

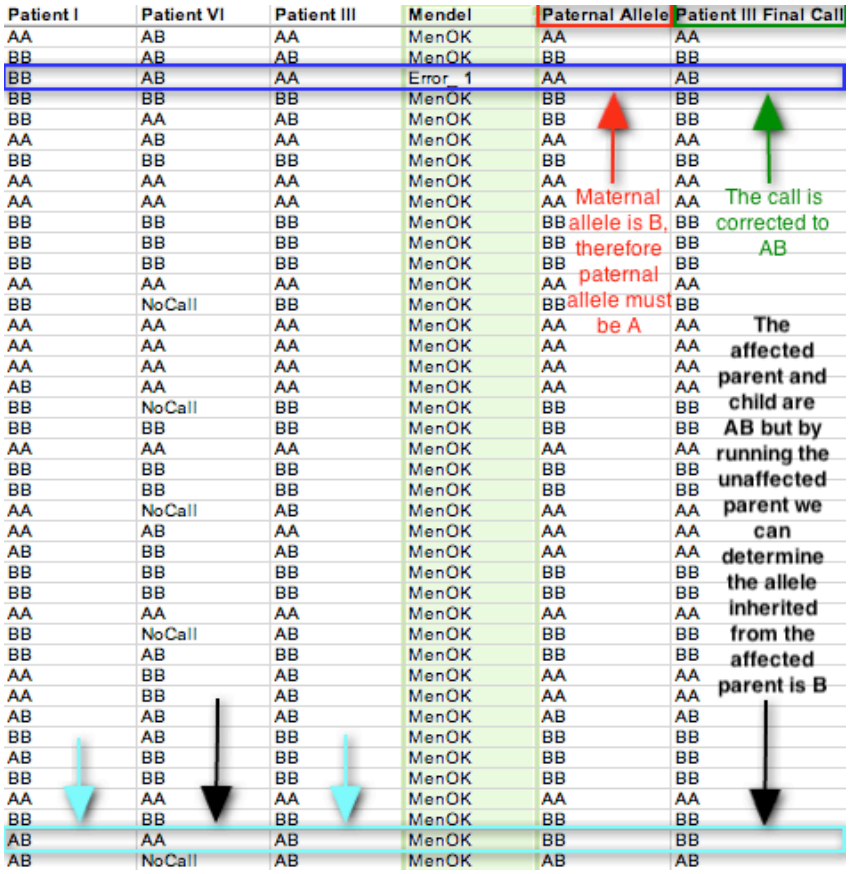


Figure 4.2 Excel image identifying a single error in Mendelian inheritance in Patient III (Patient I is the affected parent and Patient VI the unaffected parent of Patient III), the equation ADTriad then incorrectly calls the allele from the affected parent as "A" (red column) but this is then corrected to AB in the following calculation (green column). This assumes the single error is due to a miscall rather than being an actual deletion. Additionally, when both the affected parent and the affected child have the genotype AB, the allele inherited from the affected parent can be determined if the genotype of the unaffected parent is homozygous (Section 4.2.3).

Where trio data are available for a disease state and the allele of the affected parent inherited by the affected offspring has been determined and corrected as described above, then this column of alleles can be aligned with the AD exclusion data from all samples of the same disease group for comparison. The AD exclusion column can then be corrected because if at a particular SNP a consistent allele has not been determined (i.e. given the output

AB) but the Final Call data from the trio analysis have provided an output of AA (meaning A is the allele inherited from an affected parent), then the exclusion data can be changed to AA.

4.4 Copy Number Analysis

In addition to the genotyping analysis, the Affymetrix Genotyping Console can be used to investigate copy number variations in the sample genomes. For this analysis the test samples are compared to 90/270 HapMap reference samples (the number depends on the chip being used). The results are then compared to the DGV to highlight which CNVs are found in "normal" individuals and therefore highlight which may be unique to the disease.

The initial QC analysis reveals whether the data are in or out of bounds; if out of bounds the Genotyping Console analysis is suggesting the CNVs have not been identified under the desired parameters. This in/out of bounds QC is determined from the interquartile range values (IQRs) given for each chromosome (following the CNV calculations) and should be highly similar across a sample with the overall sample level IQR comparable to the individual chromosomal IQRs. If any of the values are above the user-defined threshold, then the sample is given an out of bounds output. The IQR is a measure of the dispersion/spread of the data and represents the central 50% values. It is not affected by outliers (see below) or extreme values and is therefore a robust measure of dispersion.

The 5-state Hidden Markov Model (HMM) is used for the CNV analysis where the output 0 indicates a homozygous deletion, 1 indicates a heterozygous deletion, 2 represents a normal copy number state, 3 a single copy gain and 4 represents an amplification. Various thresholds can be adjusted to analyse the

data in the best way, such as with Genomic Smoothing and Transition Decay. Smoothing adjusts the noise and if less smoothing is applied, then the variance (standard deviation, SD) of the CN states should be increased. The SD represents the underlying variance or dispersion in CN state. Transition decay is the expected correlation between adjacent SNPs because the copy number of one SNP is partially dependent on the neighbouring SNPs and weighted based on the difference between them. If the transition decay is reduced (i.e. the influence of the neighbouring SNPs) from the default of 10Mb to 1Mb then if a particular SNP has a calculated CNV of 1, the probability of the flanking SNPs also having a CNV of 1 is much lower. If a SNP has a CN state of 3 when all surrounding it are 2, then this SNP can be corrected to 2 using the 'Re-adjust outliers' option. In this project the general allele specific copy number with genomic smoothing was set at 0.5Mb with the transition decay at 10Mb and outliers re-adjusted at 1000bp.

It is important to consider the possibility of a CNV as causing a disease state because a microdeletion/duplication within or close to the disease locus may be responsible (Kohler and Cutler, 2007). Deletions in *VPS13B* have been shown to cause Cohen syndrome, an autosomal recessive disorder characterised by many features, including: retinal dystrophy, mental retardation, truncal obesity and facial dysmorphism (Balikova et al., 2009).

4.5 QPCR Analysis

In this project, quantitative polymerase chain reactions were conducted to investigate copy number variations in genomic DNA samples using a BIO-RAD C1000 Thermal Cycler. QPCR is a typical PCR reaction except an additional probe (TaqMan) or dye (SYBR Green) is added that allows detection of the amplified

product following each round of PCR. In SYBR Green reactions, the normal PCR components are used but with the additional dye SYBR Green. This dye binds to double-stranded DNA so that in each amplification cycle the dye binds to the new product. This method does have issues however, as it also encourages non-specific binding of SYBR Green. The dye will bind to any double-stranded DNA present in the reaction mixture, such as if the primers bind non-specifically, form primer-dimers or if the DNA template forms secondary structures. The TaqMan method avoids these issues as it is highly specific and the signal produced is based only on amplicon formation in each round of PCR. This method requires the design of a probe (in addition to primers) that binds within the amplified fragment. The probe contains a dye and a quencher and this latter part prevents the dye emitting fluorescence whilst in its normal state. However, once the probe is bound to the template fragment being amplified, the polymerase enzyme works its way along template forming the new strand and as it reaches the probe it begins digesting it. As the 5' end of the probe is digested the quencher is released, this means it no longer suppresses the dye and so fluorescence is given off. As fluorescence is only released as a new strand is amplified, the signal detected is directly proportional to the amount of product.

The fluorescence data generated during a QPCR can be used to compare samples by looking at the PCR cycles (Ct) necessary to achieve a given level fluorescence. Ct is proportional to the initial amount of target in the sample and the relative concentration of one target compared to another is reflected in the difference in cycle number (delta-Ct) necessary to achieve the same level of fluorescence. The Ct value is taken from the exponential phase of the reaction. The initial cycles of QPCR provide a low signal that is usually too weak to register above background until after at least

15 cycles. During the exponential phase, fluorescence doubles at each cycle. After around 35 cycles the fluorescence reaches a plateau, which indicates saturation of the reaction.

Delta-Ct is calculated by deducting the Ct of a reference target region from the Ct of the target region. A delta-Ct of 0 represents a ratio of one between the target and reference sample. Using a "normal" DNA sample control as well as test samples provides a reference for calculating delta-delta-Ct. This is done by deducting the delta-Ct for the control DNA sample from the delta-Ct of the test sample. This is the analysis process used in this project. ANOVA (analysis of variance) was used to compare the control sample values to the test group. When a significant difference was achieved, two-tailed t-tests were conducted between the control and individual test samples to determine the significant difference between the selected samples.

The reference gene for CNV analysis can be a housekeeping gene for which it would be expected that every individual would maintain two copies. *GAPDH* was chosen for this (Figure 4.3) but in future it would be best to find a different housekeeping reference gene as Redon et al. (2006) showed that one of 270 HapMap samples showed a gain/loss at the *GAPDH* genomic region in the DGV genome build 37 (Figure 4.4). However, just one of 270 would suggest this CNV is uncommon.

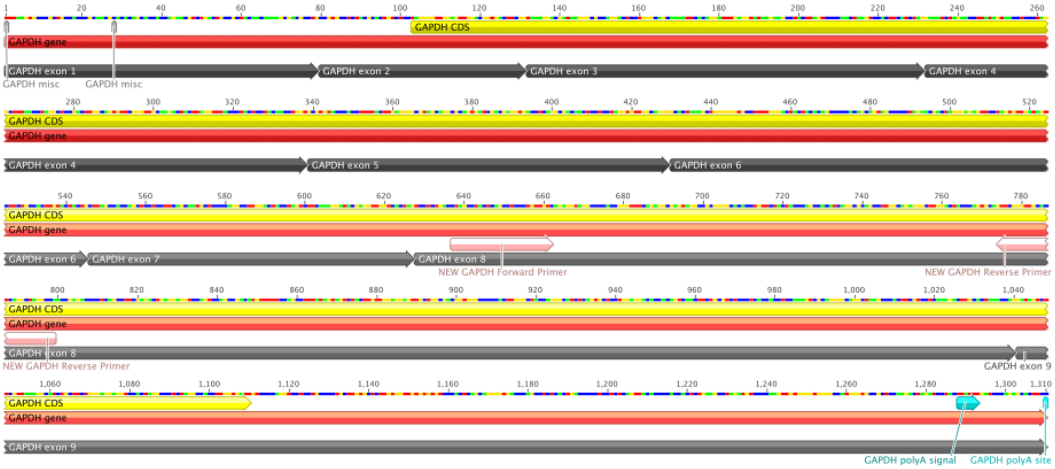


Figure 4.3 Image showing the *GAPDH* primers were designed to bind in exon 8.

4.6 Other Computational Analysis

A number of criteria can be set for the identification of candidate genes within a disease region. The genes within the disease region for the different disease states can be compared to see if they have similar functions or are members of the same gene/protein family. Given the similarity between the phenotypes of the disorders under investigation, it is possible that genes of similar function may be causing the disease states. Details of the expression of candidate genes is also important in prioritising the screening of candidate genes, for example, many of the disorders are early-onset with phenotypic manifestations for the majority being localised to the macula. Thus, when conducting bioinformatics analysis of candidate genes, the list may be refined by focussing on those expressed in the retina and during development. For MCDR4, genes expressed in both the retina and ear will be of key interest.

The sites Ensembl (http://www.ensembl.org/index.html), DGV (http://projects.tcag.ca/variation/), NEIBank (http://neibank.nei.nih.gov/EyeSAGE/index.shtml) and NCBI

(http://www.ncbi.nlm.nih.gov/) were extensively used in this project for bioinformatics analysis. These sites were used to investigate SNP location and information and candidate gene investigations. The software Geneious (Drummond et al., 2010) was also used extensively and throughout the project much time was spent investigating candidates and using these databases in addition to the Affymetrix Genotyping Console.

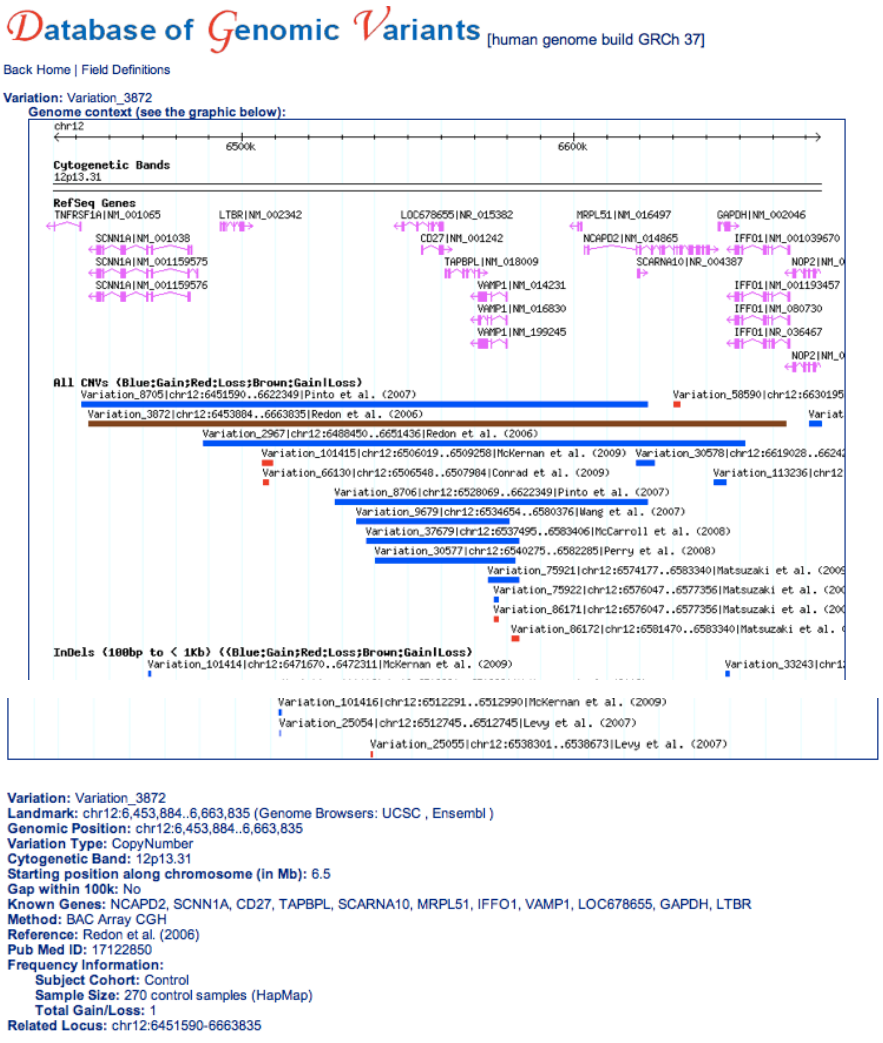


Figure 4.4 Image taken from the DGV genome build 37 showing the *GAPDH* CNV identified by Redon et al. (2006).

5. NORTH CAROLINA MACULAR DYSTROPHY (NCMD/MCDR1)

5.1 MCDR1 Genetic Analysis

The disorder is well known amongst scientists and ophthalmologists due to all the years it has been known about yet the genetic cause remains elusive. It is a non-heterogeneous disorder with all families tested linking to the same locus on 6q (Small, 1998). Interestingly, in recent years two new disorders, MCDR3 and MCDR4, have been identified with an almost identical ocular phenotype yet they do not link to 6q. It may be that similar genes are involved or that the genes affected in each disorder are involved in the same process/function/pathway. It is also of interest that PBCRA links to an overlapping region on 6q so it is possible that the same gene is affected in both conditions but in different ways. Either way, discovering the cause of MCDR1 will provide a better understanding of macular function and dysfunction.

The phenotype of MCDR1 is described in Section 2.5.1 but briefly: patients have a stable condition that varies between members of the same family in its severity. The phenotype is only unstable if there is formation of SRNVM in Grade III MCDR1. Patients lose central vision and though visual acuity can be good, with Grades II and III vision is poor and cannot be recovered. If a mutation can be identified in a particular gene then the possibility of gene therapy arises. If a CNV increase appears to be causing the disease state then it will be more difficult to find effective treatment, whereas a loss of a gene can also be treated with gene therapy.

MCDR1 was originally mapped by Small et al. (1992b) to chromosome 6q16 and the disease locus named by the Human Genome Organisation as MCDR1 (MC = macular, D = dystrophy, R = retinal, 1 = first macular degeneration to be mapped). Small (1998) and Small et al. (1999) later reported on the various linkage studies that have been conducted with NCMD families and produced Figure 5.1 in summation of these investigations. Haplotype analysis revealed four families from the USA shared the same haplotype whilst families from Belize, Britain, France and another two from America appeared to have separate haplotypes, suggesting independent mutations arose in these families. The MCDR1 locus was determined to lie between the markers D6S249 and D6S1671.

More recently, the disease region has been refined by Yang et al. (2007) using six families exhibiting the NCMD phenotype and in which the disorder was linked to the MCDR1 locus. This group refined the disease interval to a 1.8 million bp region from 6q16.1 to 6q16.2 between markers D6S1716 and D6S1671. Eleven of the genes annotated at the time within this region (eight of which are indicated in Table 5.1) were analysed by mutation screening within the coding regions yet no mutations were found. D6S1716 lies between the first two annotations listed in Table 5.1 whilst D6S1671 lies just after the last annotation in the table. The highest scoring marker within this region was D6S1717, which lies ~100kb upstream of C6orf168. Whilst detailed linkage analysis has been conducted on numerous MCDR1 families and CDS within the locus screened for mutations, CNV analysis has never been reported and will be conducted in this project.

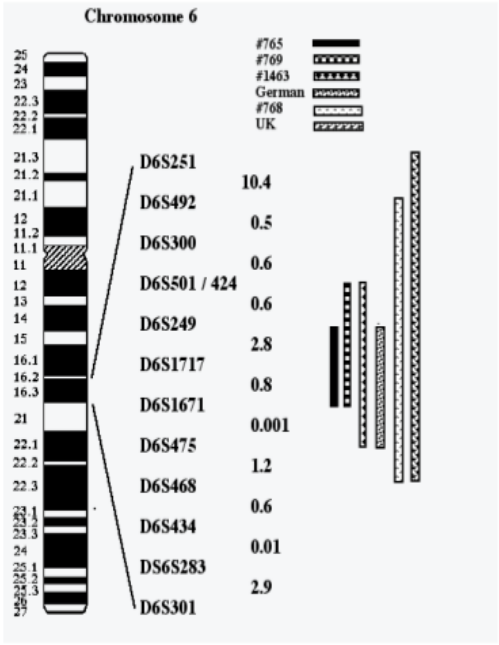


Figure 5.1 Ideogram of chromosome 6 showing the location of markers and distances between them in centiMorgans. The location of the MCDR1 gene as determined by various family studies is shown by the shaded bars. Taken from Small et al. (1999).

Initially it was intended to conduct Affymetrix analysis using DNA from several members of the original British MCDR1 family (Figure 5.2) reported on by Reichel et al. (1998). However, permission to use these samples was not obtained until very near the end of the project. At this point it was discovered that the DNA quality of the majority of samples was poor due to the length of time in storage and sadly it was not good enough for Affymetrix processing. Due to limited resources it was initially deemed worthwhile to analyse as many affecteds from different families as possible as opposed to parent-offspring pairs from half the number of families. The samples indicated in Figures 5.2 and 5.3 were analysed for CNV analysis and though the samples chosen would not allow for haplotype determination, it was thought that comparisons of the genotypes would still reveal shared regions of similarities.

Each of the families (Figure 5.3a-h) were of European origin but were small, containing only one or two affected members. For this

reason it was not appropriate to conduct linkage analysis as a number of subjects separated by at least 10 meioses are required to obtain significant LOD scores. As MCDR1 is a non-heterogeneous disorder and MCDR3 and MCDR4 are extremely rare with just a single family identified for each, it is highly likely that these NCMD families do indeed link to 6q and so analysis of data focused on this region.

From the original British MCDR1 family, two samples were of good enough quality to process on the Affymetrix chips, an unaffected parent and affected offspring (indicated in Figure 5.2) from whom the disease genotype for this family could be determined.

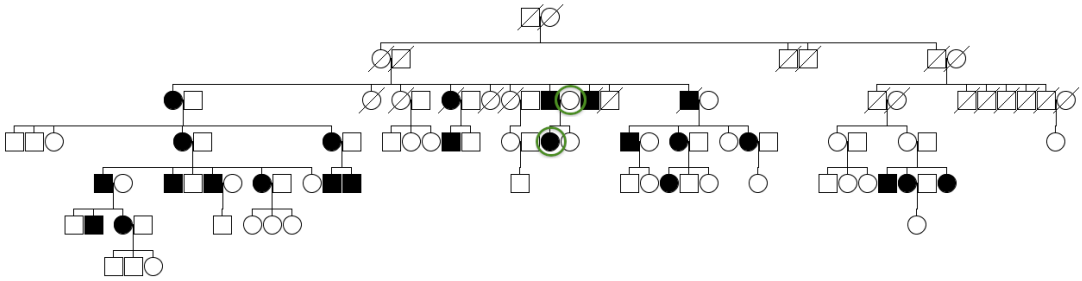
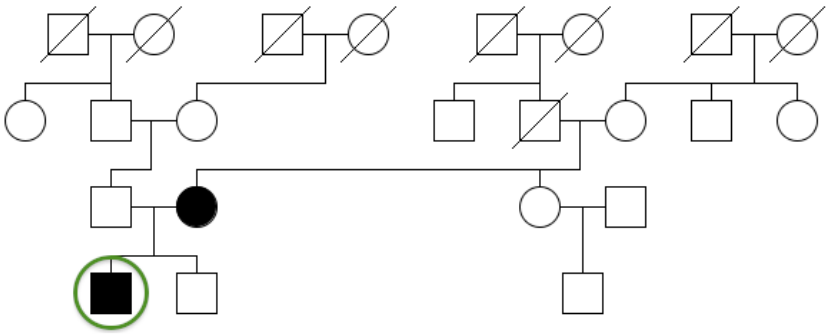
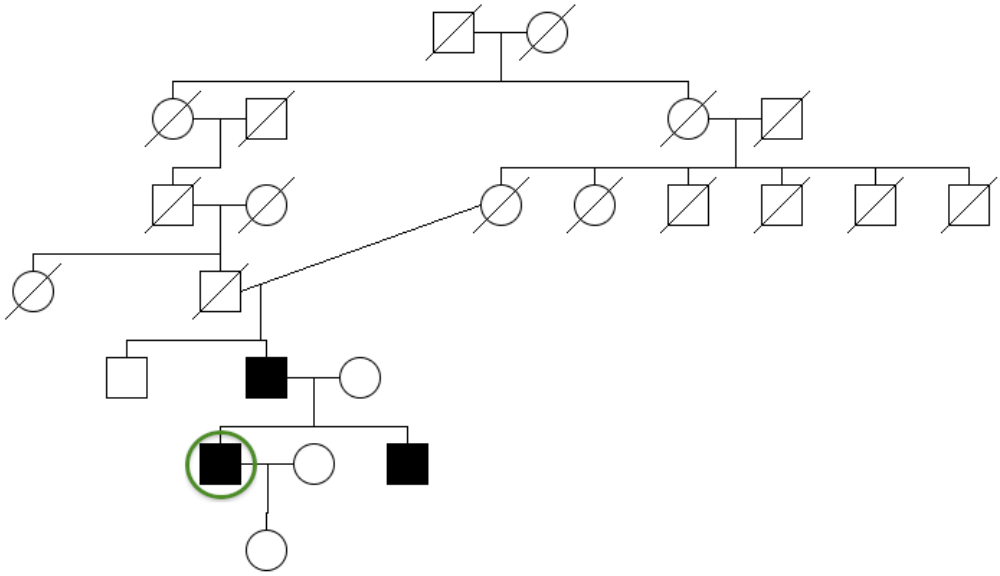


Figure 5.2 The original British MCDR1 family as reported on by Reichel et al. (1996). The individuals circled in green are those analysed on the Affymetrix 50K *Sty I* chips. Individuals highlighted in black are known affected members.

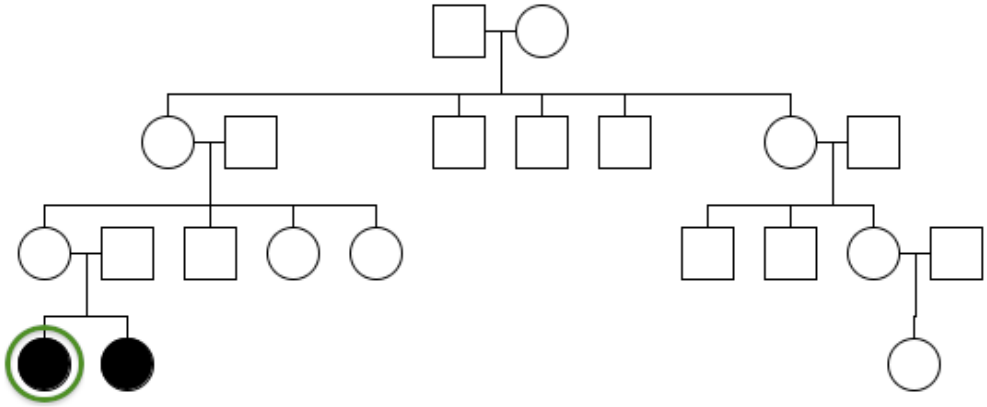
a)



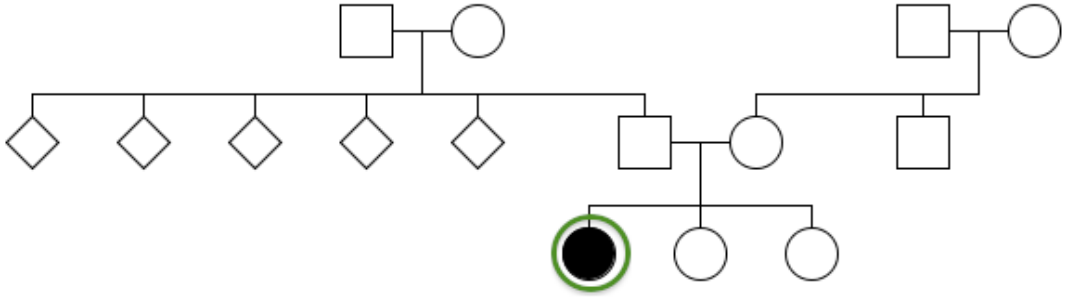
b)

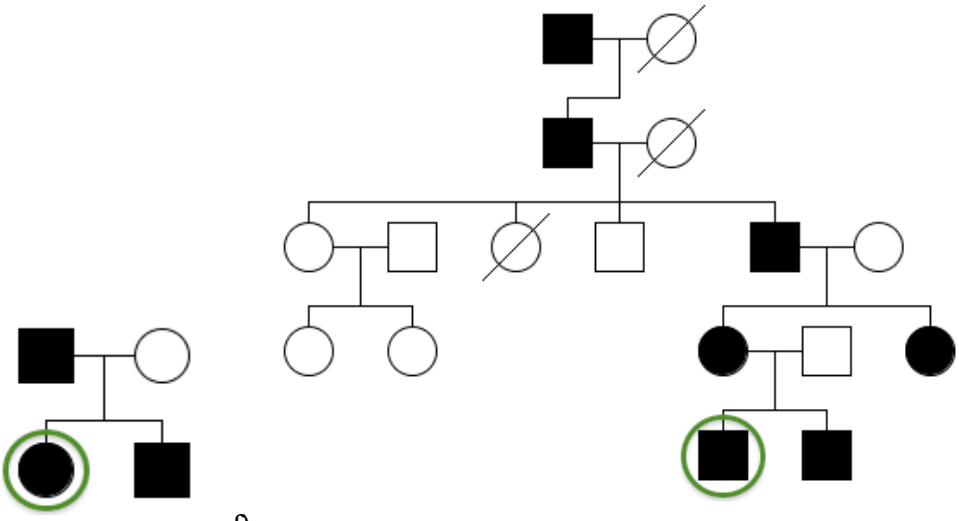


c)

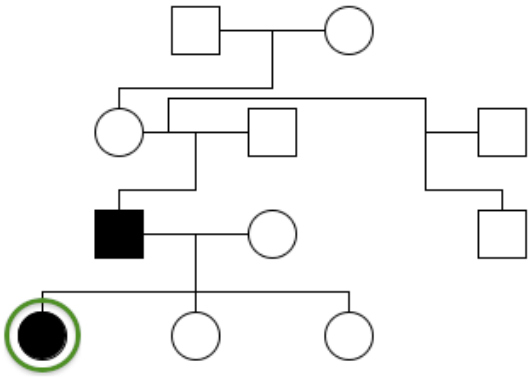


d)

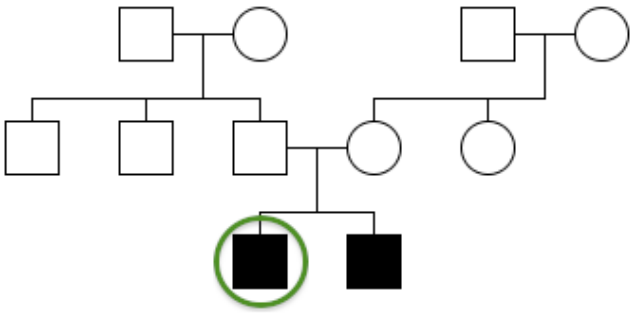




e) f)



g)



h)

Figure 5.3a-h Pedigrees of MCDR1 families with known affected members highlighted in black and individuals whose DNA was analysed on the Affymetrix 50K *Sty I* SNP chips are highlighted in green.

5.2 Candidate Genes

Despite Yang et al. (2007) screening the exons and sequencing the cDNAs of the genes within the MCDR1 locus (Table 5.1) in patients with NCMD and finding no abnormalities, these remain candidate disease genes as mutations may exist in promoter regions, at splice sites or within introns. It may also be that deletions of exons were missed due to the sequencing strategy.

Table 5.1 Annotations within the MCDR1 disease region of the human genome build 37. *cDNA screened by Yang et al. (2007).

Gene	General Function
RP11-436D23.1	Processed transcript
RP3-453D15.1	Processed transcript
RP11-67P15.1	Pseudogene
POU3F2, POU domain, class 3 homeobox 2	Transcription factor
FBXL4, F-box/ leucine-rich repeat protein 4	Involved in phosphorylation-dependent ubiquitination
RP11-758C21.1	Pseudogene
C6orf168	Uncharacterised protein coding
COQ3, coenzyme Q3 homolg	Methylates an early coenzyme Q intermediate: important in electron transport pathways
SFRS18, splicing factor, arginine/serine-rich 130	Splicing factor
RP11-9819.4	Processed transcript
USP45, ubiquitin carboxy-terminal hydrolase 45	Involved in the ubiquitin cycle
RP1-199J3.3	Processed transcript
AL137784.1	Novel misc RNA
CCNC, cyclin-C	Part of the RNA polymerase II holoenzyme complex
RP1-199J3.5	Pseudogene
PRDM13, PR domain zinc finger protein 13	Regulator of transcription
AL035087.1	Novel misc RNA
MCHR2, melanin-concentrating hormone receptor 2	G-protein coupled receptor
RP11-1414.3	Processed transcript
RP11-1414.2	Pseudogene
AL08028.5	Novel pseudogene

5.2.1 *SFRS18*

Of the genes listed in Table 5.1, *SFRS18* is the only one reported to be expressed in the retina and RPE/choroid (NEIBank database). It encodes the serine-arginine-rich-splicing regulatory protein 130 (SRrp130). Zimowska et al. (2003) reported this to be a novel 130 kDa nuclear protein consisting of 805 amino acid residues with multiple serine-arginine (SR) repeats that co-localises with *Pnn* in a human corneal epithelial cell line. *Pnn* encodes pinin, a phosphoprotein thought to play a key role in the establishment and maintenance of corneal epithelial adhesion (Shi et al., 2000). SRrp130 exhibits an SR-rich carboxy-terminus, which caused the authors of the study to speculate the involvement of SRrp130 in pre-mRNA biogenesis through interactions with SR-rich proteins. SRrp86 is known to be involved in activation and inhibition of splicing (Barnard and Patton, 2000) and the C-terminus of SRrp130 shows significant homology to SRrp86. There have been no reports to date of a role of this gene in retinal function or development but NEIBank Database lists it as having expression in the retina. As it may play a role in splicing, it is possible that NCMD is caused by a novel faulty splicing mechanism due to a mutation in this gene. Various mutations affecting splicing have been found to cause RP (Section 2) so it is plausible to consider such a mechanism for macular dystrophy.

5.2.2 *POU3F2*

POU domain class 3, transcription factor 2 gene (*POU3F2,* also known as *BRN2,* brain-specific homeobox/POU domain protein 2) belongs to a large family of transcription factors that bind to an octameric DNA sequence with the class III POU genes expressed predominantly in the central nervous system. *POU3F2* is involved

in embryonic neuronal development and Cobrinik et al. (2006) reported that the protein product of this gene binds to promoters of characteristic retinal progenitor cells (RPCs) and is down-regulated by retinoblastoma protein (Rb). During retinal development it is expressed in intermediate and late RPCs as well as in post-mitotic cells. As this gene has been identified as being active during the development of the retina, it may be a stronger candidate than *SFRS18* for NCMD. It is likely to bind to promoters for many retinal genes, for example, GeneCards indicates the ABCA4 promoter has a POU3F2 site (http://www.genecards.org/cgi-bin/carddisp.pl?gene=ABCA4). If it is important for the expression of retinal genes then a mutation could clearly have a crucial impact on the functioning of the retina.

5.2.3 *FBXL4*

The F-box/leucine-rich repeat protein 4 (encoded by *FBXL4*) is a component of modular E3 ubiquitin protein ligases, which function in phosphorylation-dependent ubiquitination. The fly homologue, *dFbxl4,* is dependent upon fly calmodulin-binding transcription activator (*dCAMTA*) for expression and together they facilitate rhodopsin deactivation (Han et al., 2006). It is suggested that *dFbxl4*-mediated ubiquitination of rhodopsin may abolish rhodopsin-Gq interaction and so deactivate the light receptor in *Drosophila*. Loss of *dCAMTA*/*dFbxl4* function causes rhodopsin to undergo light-dependent down-regulation. Thus, it appears that in flies, *dFbxl4* is important for sensitivity and termination of the light receptor. The importance of *dFbxl4* in *Drosophila* phototransduction may suggest a similar role of *FBXL4* in human retina. If the human homologue does play a similar role in the human retina then this is a good candidate gene for NCMD.

5.2.4 Other Candidate Genes

Neither *CCNC* nor the methyltransferase *COQ3* have been reported to be involved in retina-specific functions or development so they would not appear to be good candidates for NCMD. Similarly, *PRDM13* is not a high priority candidate gene in NCMD but it has been considered as a candidate tumour suppressor gene (Behrends et al., 2003). MCHR2 is a G-protein-coupled receptor activated by melanin concentrating hormone and is involved in appetite regulation (Hill et al., 2001). This gene is an intriguing candidate as there is no orthologue present in mice and, as mice lack a macula, it is possible the *MCHR2* gene has an as yet unknown role in macular function. Dr Ambreen Kalhoro has previously screened the coding exons of this gene in MCDR1 patients but failed to find a mutation (unpublished data).

5.3 Methods Applied

5.3.1 Affymetrix 50K *Xba* I Protocol

The DNA samples were prepared for processing over the Affymetrix 50K *Xba* I chip, as described in Section 3.2.2. Hybridisation to the chips was conducted by the UCL Wolfson Institute.

5.3.2 Candidate Screening

FBXL4

Gradient BIOTaq PCRs were conducted for all primers (see Section 3.1.1) and PCRs then carried out at optimal temperatures for each primer set using the BIOTaq protocol. Primers and annealing temperatures are shown in Table 5.2.

Table 5.2 *FBXL4* primers and annealing temperatures.

Primers	Name	Annealing Temperature (°C)
GAGATCTACCTTGATTCCTCGAAG	AF	64
CTACTCAGGATGGTATCTTAAGGC	AR	
AATGTGGCCTGTTTGATTTGAAG	2F	64
CTTTGGTGCTGACAGTTAATCC	NEW 2R	
GCCTGTTCATTACCTTATCCATG	3F	59
ACAGTATGAGGTATTACAGGTATA	NEW 3R	
GGTCTATAACCTTAAGGGACCAG	4F	60
TGCTCAATTACCGATGCTCAGT	4R	
CATAGAATGATACTGCTTTTCAC	5F	60
CAGCATTCTAAACATGACACTCA	5R	
CAAATGTATCTACTTAGAGTTGC	6F	60
GTCAGAAGGCATCATAAACTTGA	6R	
AGCAGTGACAGCCTTTAATGTCTA	7F	60
AGACATACTTTCCAATTTAAGA	7R	
CTTACTCTAAACAGTTTGCATGTC	8F	64
ACCAAGCCATCTTATCAGGATAA	8R	
CAATAAATCATGGATGGATTCAT	9AF	64
CACTCATATTCTTGAAGAGAAGT	9AR	
TTCATAAAATACTGCAAGCACTT	9BF	64
GTGCAATCATAAGTACACTAGAG	9BR	
CTCTAGTGTACTTATGATTGCAC	BF	60
AATGTTAAGGCATCTGCTTCTA	BR	

SIM1

Gradient BIOTaq PCRs were conducted for all primer sets (Section 3.1.1) and PCRs then carried out at optimal annealing temperatures for each primer set. The BIOTaq protocol was followed and amplification products used directly in sequencing reactions unless otherwise stated (Table 5.3).

Table 5.3 *SIM1* primers and annealing temperatures.

Primers	Name	Annealing Temperature (°C)
TAACTCCCCAAACCGGCCT	Another AF	62, GoTaq, gel-extracted
GTGACACTTACACACCAAG	Alternative AR	
TGGAATTGGGACGAGTGTGTGAG	A1F	64
CAAAGTCACTTACCTTCTGGGAAC	A1R	
CAGGTCCGGGTTCAGTGG	2F	63, GoTaq, gel-extracted
TTGCAGCAGCTATTGGGCTCT	A2R	
AGTCACTAACTTCTGTCCTCC	A3F	64
GAGAGACCCAGAACTATTTAAG	A3R	
CCACGGCGACGGCGACATC	4F	65 (montage clean-up)
CTTGCTTCCCGCCTCCTCTGACTC	5R	
TGTGGCTGAGTCTCCCTCCCTATC	6F	64
CAGCGGATGCGCCAAGGTTG	7R	
AGGGCTCGATGCAATACCACGGGA	A8F	64
GCCCGAGCCTTGTCTAACCCGGC	A8R	
ATGGTGGCTGATTAAGGGCTTTGT	9F	64
AGAGAACCTTCCAGATTTATA	A9R	
CAATGAGACCTTAAGGGTGCTTGTAG	10F	64
TGGAGTTCGGGAACCCTTTCAC	10R	
CCAGTACAGAGAGTTGACAC	A11FA	64
CATAGTAAATGCTGGTAATGGGGTAT	11RA	
ATACCCCATTACCAGCATTTACTATG	11FB	64
TATGCTGAGCCCTTAAATTGT	11RB	
ACAATTTAAGGGCTCAGCATA	11FC	64
CCTGAGTATTCAATTTCATGTTC	11RC	

Novel Putative mRNA Transcript

Two predicted exons of a putative, incomplete mRNA transcript were screened using the BIOTaq protocol. An annealing temperature of 56°C was used for both exons and the sequences

were cleaned using the Montage method (Section 3.1.1) prior to analysing. Primers are listed in Table 5.4.

Table 5.4 Primers used for the amplification of a novel putative mRNA transcript.

Primers	Name
TTGAAATAAGCAGAGGATTG	S1NG1F
TCTAGGCAGAGGCAATGTATGT	S1NG1R
GTCCATAGTCACTGGGCTAAT	S1NG2F
AGAGGGAGAAACGTCAGCA	S1NG2R

POU3F2

Numerous attempts to screen *POU3F2* were made yet all failed. Four different polymerase enzymes were tried and numerous primers designed (Figure 5.4 and Table 5.5). Manually designed primers were used in addition to software-designed primers. Attempts were made to amplify the entire length of the gene in addition to amplifying it in sections. None of these attempts were able to provide sequencing data.

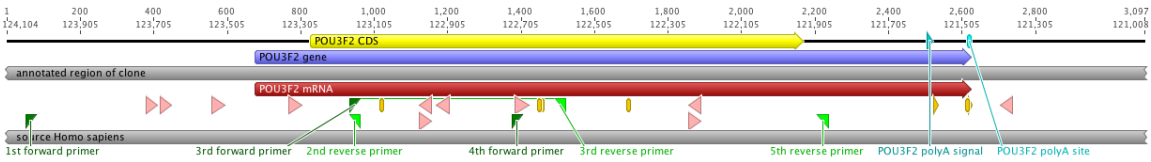


Figure 5.4 Image showing the positions of primers designed and used in attempts to amplify *POU3F2*.

Table 5.5 Primers used in attempts to screen *POU3F2*. Names of primers match those annotated in Figure 5.3 from left to right.

Primers	Name
GCTCGGTGCGACCCAGCTTT	1st Forward
GAGTAGCTCTGCGCCAATCAG	Amplicon 1F - NEW
CGCCGAGCTAGTCAGAGAGTGAGC	Forward Primer 1F
GCGCCGAGCTAGTCAGAGAGTGAGC	Old Primer 1F
AAGTAACTGTCAAATGCGCGGCTCCT	Before CDS F
CTGCACCAGGCTCTGCGCTT	2nd Reverse
AAGCGCAGAGCCTGGTGCAG	3rd Forward
CCGAGGGCTTGATGTCCGGCTGGC	Amplicon 1R
GCCAGCCGGACATCAAGCCCTCGG	Amplicon 2F
TGGCCCGTGCAGCTCGTCTCCG	NEW 1R
GTCCAACGGCGGCTTGCTCT	4th Forward
CGGCTTGCTCTACTCGCAGCCCAGCTTC	NEW 2F
GGGTGGTGGTCGGCATGGTG	3rd Reverse
CTTGCGCTGCGATCTTGTCTATGC	Amplicon 2R - NEW
GCATAGACAAGATCGCAGCGCAAG	Amplicon 3F
GCCGGGAAAGGGCCAAGGAC	5th Reverse
TATGCTCATGAATTTGTTCACGT	Amplicon 3R - NEW

5.4 Results

5.4.1 Affymetrix Data

An affected individual from each of nine unrelated families was selected for screening in order to analyse genotypes at the MCDR1 disease interval between D6S1716 and D6S1671. An unaffected member of the original MCDR1 family (Reichel et al., 1998) and her affected offspring were analysed for genotype comparison. The aims of this work were to ascertain whether the families possess disease regions with common alleles and to determine whether there is any evidence for chromosomal deletion or duplication within the MCDR1 region.

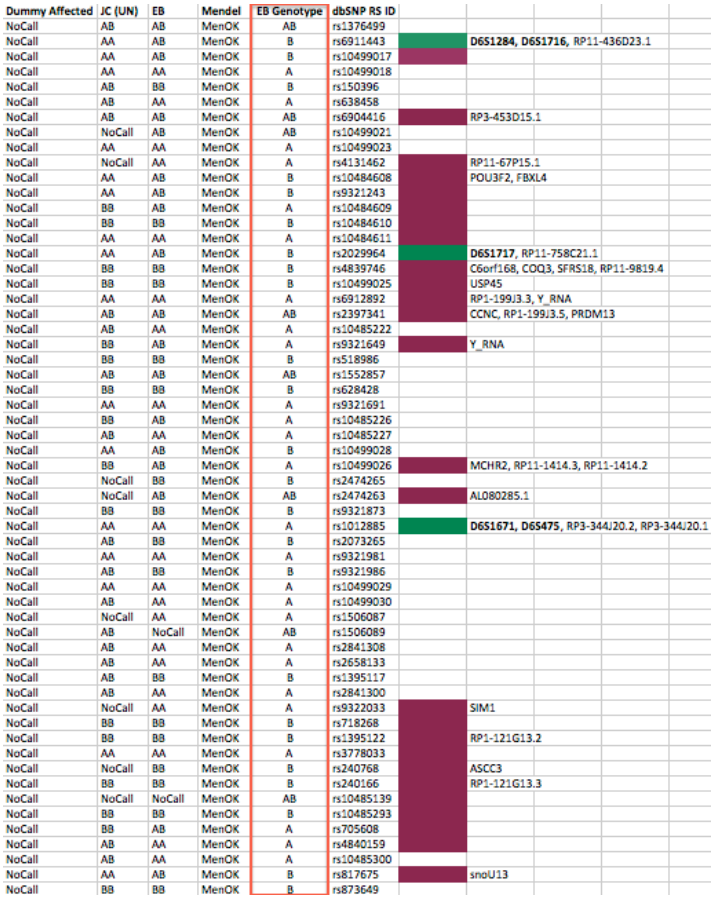
Genotyping Analysis

The Affymetrix Genotyping Console determined all samples to be of good quality (90 - 96% QC rate), indicating the genotyping results generated would be reliable.

The data from the original British MCDR1 family members were used to determine a MCDR1 genotype at the 6q locus. This is the only family in this study that has been linked to chromosome 6q (Reichel et al., 1998). The genotypes from the unaffected subject were compared to those of the affected offspring and the allele inherited from the affected parent deduced (as described in Section 4.3.2), producing the disease genotype for this family (Figure 5.5). Where AB is given, this indicates that the disease allele could not be determined. The genotype for the original British MCDR1 family was then aligned against the other nine MCDR1 sample data for comparison (Figure 5.6). It should be noted that in this comparison the disease genotype for the original MCDR1 family identifies the disease alleles A or B as AA or BB, respectively.

A consequence with not running unaffecteds from each family is that it does not allow the determination of phase for the unrelated families. The Affymetrix Console provides calls only for the alleles it detects, it cannot identify which allele is from the maternal or paternal chromosome and therefore the haplotypes cannot be determined without analysing both parent and offspring samples. For this reason, running either one parent of each affected or an unaffected sample for each family would have been ideal. However, this was only done for the original British family. For the new families it was initially deemed worthwhile with limited

resources to analyse affecteds from as many families as possible rather than parent-offspring pairs from fewer families. Parent-offspring data would have determined haplotypes but instead only comparison of the genotypes within the disease locus could be conducted. It was realised during the analysis process that the choice of samples for analysis was ill-judged. Figure 5.6 shows the comparison between the MCDR1 6q genotype of the original British family to the same region in affected samples from unrelated families.



Dummy Affected	JC (UN)	EB	Mendel	EB Genotype	dbSNP RS ID	
NoCall	AB	AB	MenOK	AB	rs1376499	
NoCall	AA	AB	MenOK	B	rs6911443	**D6S1284, D6S1716,** RP11-436D23.1
NoCall	AA	AB	MenOK	B	rs10499017	
NoCall	AA	AA	MenOK	A	rs10499018	
NoCall	AB	BB	MenOK	B	rs150396	
NoCall	AB	AA	MenOK	A	rs638458	
NoCall	AB	AB	MenOK	AB	rs6904416	RP3-453D15.1
NoCall	NoCall	AB	MenOK	AB	rs10499021	
NoCall	AA	AA	MenOK	A	rs10499023	
NoCall	NoCall	AA	MenOK	A	rs4131462	RP11-67P15.1
NoCall	AA	AB	MenOK	B	rs10484608	POU3F2, FBXL4
NoCall	AA	AB	MenOK	B	rs9321243	
NoCall	BB	AB	MenOK	A	rs10484609	
NoCall	BB	BB	MenOK	B	rs10484610	
NoCall	AA	AA	MenOK	A	rs10484611	
NoCall	AA	AB	MenOK	B	rs2029964	**D6S1717,** RP11-758C21.1
NoCall	BB	BB	MenOK	B	rs4839746	C6orf168, COQ3, SFRS18, RP11-9819.4
NoCall	BB	BB	MenOK	B	rs10499025	USP45
NoCall	AA	AA	MenOK	A	rs6912892	RP1-199J3.3, Y_RNA
NoCall	AB	AB	MenOK	AB	rs2397341	CCNC, RP1-199J3.5, PRDM13
NoCall	AB	AA	MenOK	A	rs10485222	
NoCall	BB	AB	MenOK	A	rs9321649	Y_RNA
NoCall	BB	BB	MenOK	B	rs518986	
NoCall	AB	AB	MenOK	AB	rs1552857	
NoCall	BB	BB	MenOK	B	rs628428	
NoCall	AA	AA	MenOK	A	rs9321691	
NoCall	BB	AB	MenOK	A	rs10485226	
NoCall	AB	AA	MenOK	A	rs10485227	
NoCall	AA	AB	MenOK	B	rs10499028	
NoCall	BB	AB	MenOK	A	rs10499026	MCHR2, RP11-1414.3, RP11-1414.2
NoCall	NoCall	BB	MenOK	B	rs2474265	
NoCall	NoCall	AB	MenOK	AB	rs2474263	AL080285.1
NoCall	BB	BB	MenOK	B	rs9321873	
NoCall	AA	AA	MenOK	A	rs1012885	**D6S1671, D6S475,** RP3-344J20.2, RP3-344J20.1
NoCall	AB	BB	MenOK	B	rs2073265	
NoCall	AA	AA	MenOK	A	rs9321981	
NoCall	AB	BB	MenOK	B	rs9321986	
NoCall	AA	AA	MenOK	A	rs10499029	
NoCall	AB	AA	MenOK	A	rs10499030	
NoCall	NoCall	AA	MenOK	A	rs1506087	
NoCall	AB	NoCall	MenOK	AB	rs1506089	
NoCall	AB	AA	MenOK	A	rs2841308	
NoCall	AB	AA	MenOK	A	rs2658133	
NoCall	AB	BB	MenOK	B	rs1395117	
NoCall	AB	AA	MenOK	A	rs2841300	
NoCall	NoCall	AA	MenOK	A	rs9322033	SIM1
NoCall	BB	BB	MenOK	B	rs718268	
NoCall	BB	BB	MenOK	B	rs1395122	RP1-121G13.2
NoCall	AA	AA	MenOK	A	rs3778033	
NoCall	NoCall	BB	MenOK	B	rs240768	ASCC3
NoCall	BB	BB	MenOK	B	rs240166	RP1-121G13.3
NoCall	NoCall	NoCall	MenOK	AB	rs10485139	
NoCall	BB	BB	MenOK	B	rs10485293	
NoCall	BB	AB	MenOK	A	rs705608	
NoCall	AB	AA	MenOK	A	rs4840159	
NoCall	AB	AA	MenOK	A	rs10485300	
NoCall	AA	AB	MenOK	B	rs817675	snoU13
NoCall	BB	BB	MenOK	B	rs873649	

Figure 5.5 Excel sheet extract showing the Mendel and ADTriad comparisons between the original British family unaffected parent and affected offspring. A Dummy Affected parent is added for the Macro equations to function. The determined disease genotype of the affected offspring is boxed in red.

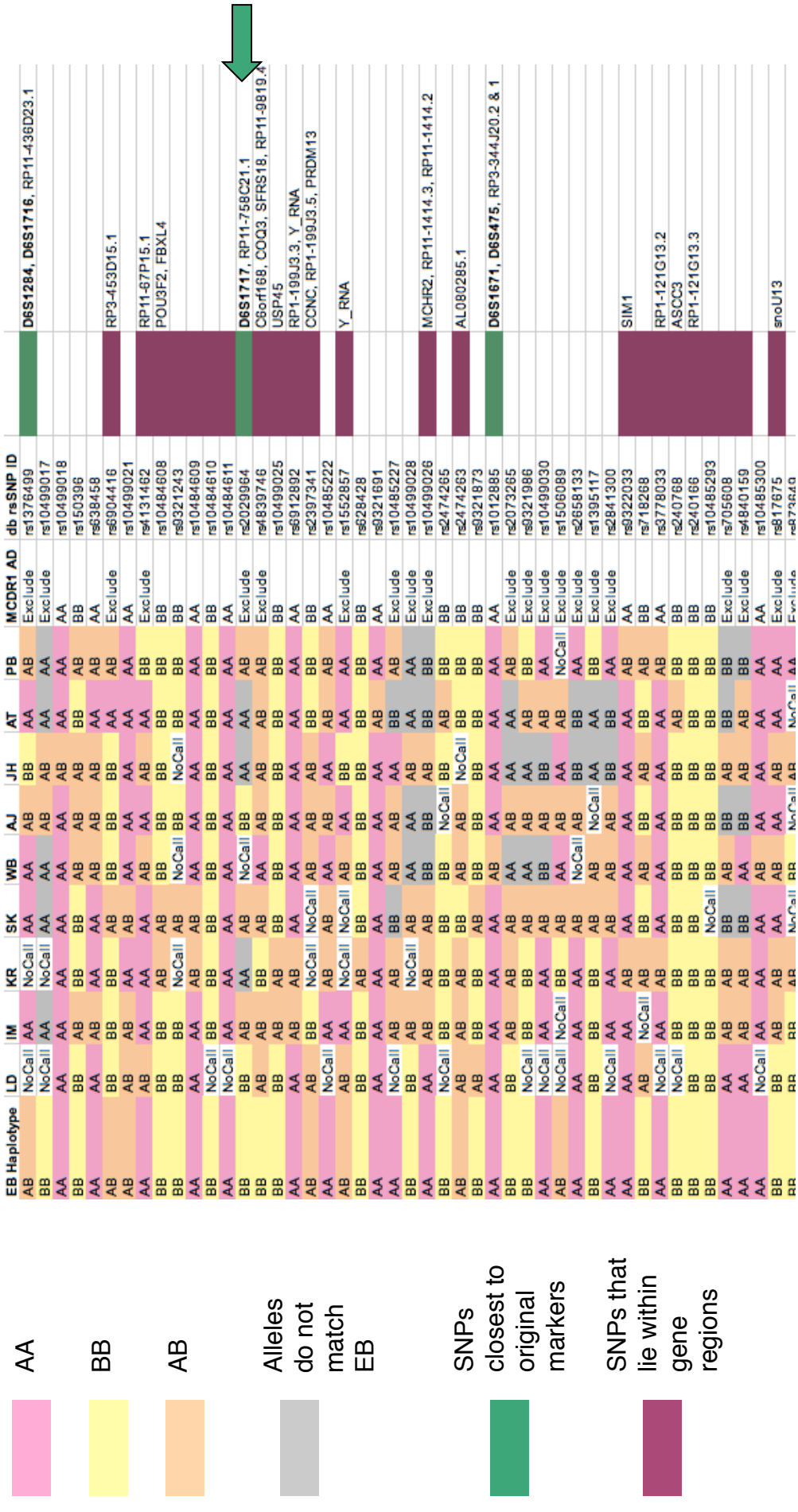


Figure 5.6 50K SNP data genotypes for 10 families across the 6q16 MCDR locus. The arrow indicates the marker that has achieved the highest LOD scores.

Though the comparisons of the unrelated families are not ideal, when aligned with the genotype of the orginal British family, there are apparent regions of common alleles within the disease locus (Figure 5.6). For example, around the highest scoring microsatellite marker D6S1717 are the greatest number of shared alleles between all samples. The genes within this region are: *POU3F2, FBXL4, USP45, CCNC* and *PRDM13*. However, this approach is not ideal. Not knowing the phase of each allele means it cannot be known which allele is on the disease chromosome. There are many SNPs for which AB calls are provided and it may be that even though all samples appear to have a common allele, for some samples the shared allele may not be part of the disease haplotype.

Another issue arising from the approach taken is that SNPs on the Affymetrix chips tend to be common SNPs and so the similarities identified by comparing the ten unrelated MCDR1 families may be seen by comparing any 10 unrelated samples. To highlight this, the genotypes of 10 randomly selected HapMap samples were aligned and compared at the 6q locus in the same manner as for the MCDR1 samples in Figure 5.6. These are ten unrelated families as in the MCDR1 analysis and comparing the alleles across the 6q locus produces a very similar exclusion pattern as seen from the MCDR1 sample comparisons (Figure 5.7).

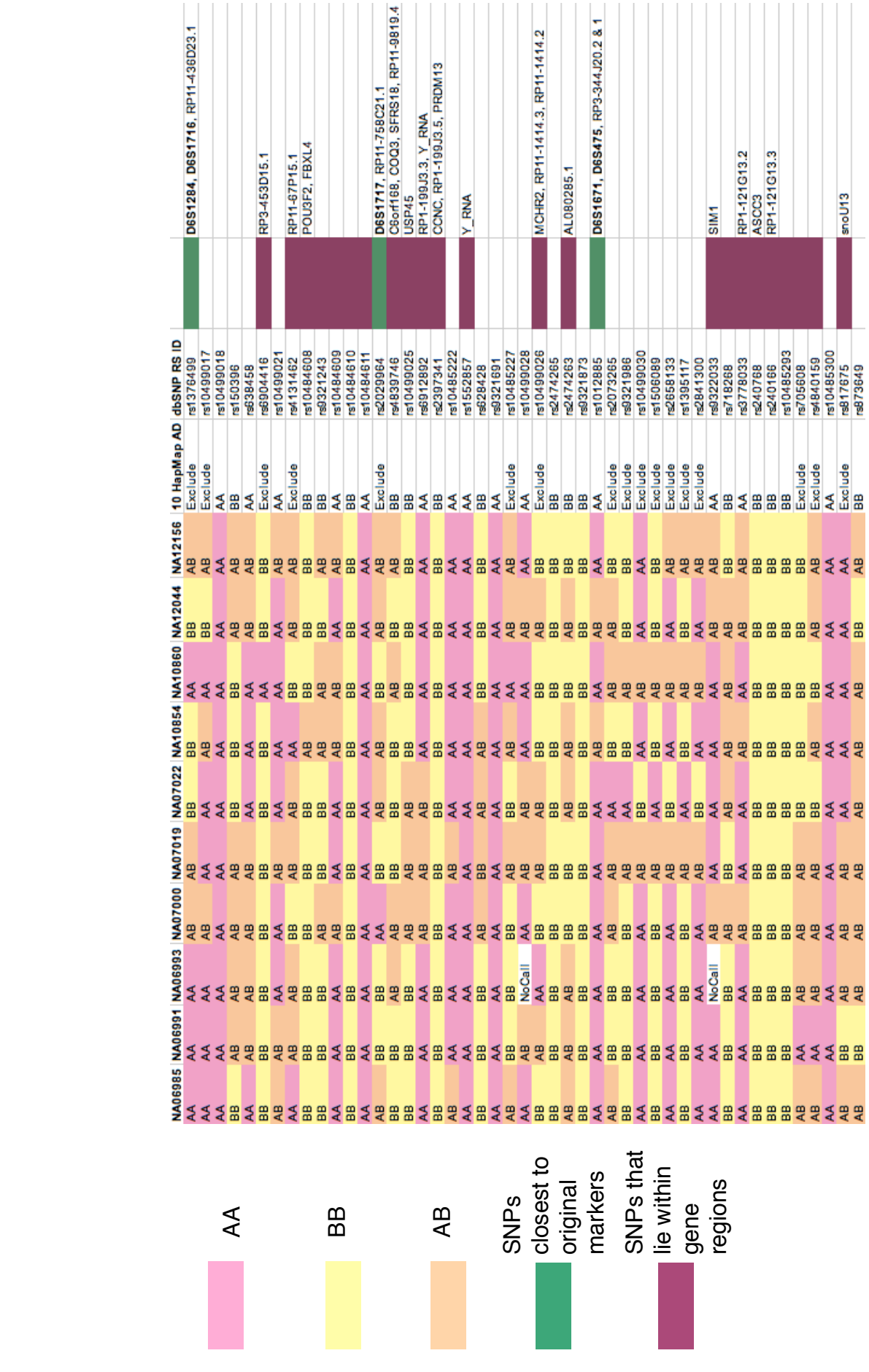


Figure 5.7 50K SNP data genotype for 10 random HapMap samples across the 6q16 MCDR locus.

Copy Number Analysis

The 50K chip data is not ideal for CNV analysis as the 250K and SNP 6.0 chips provide greater analysing capacities and more reliable outputs. Despite this the data set was compared to 90 normal samples (HapMap) to give an indication of possible regions of CNV in each sample that could then be compared and investigated further. The quality of the sample data were adequate for the analysis, with all sample data determined to be in-bounds. All CNVs were compared to the DGV for identification of novel CNVs.

For all samples, the analysis indicated that every chromosome contained a number of CNVs. This contrasted with the analysis of sample HapMap data and the analyses from other components of the project where CNV analysis was conducted using 250K data. The 50K chip would appear to be identifying spurious CNVs within the genome. Though all families in this analysis have not been linked to the MCDR1 locus, it is likely that they are MCDR1 families and therefore a common variation would be expected. However, none of the CNVs identified were found in all families, as shown in Table 5.6. This suggests that all the NCMD families do not share a common CNV but given the reliability of the data, the CNV discovered in seven of the ten families across *POU3F2* and *FBXL4* should be investigated further.

Table 5.6 CNVs not found in the DGV but common to the families analysed.

CN State	First SNP	Last SNP	Length (bp)	Within the CNV	Samples with the CNV
3	rs10485380	rs10499012	274,554		AJ, AT, IM, JH, KR, LD, PB, WB
1	rs1872841	rs10499018	196,280		AJ, JH, LD, PB, SK, WB
3	rs210399	rs2067012	555,451	*POU3F2, FBXL4*	AT, IM, KR, LD, PB, SK, WB

5.4.2 Candidate Screening

Though previous screens of patient cDNA have revealed no mutations, the cause of disease could be a splice variant of a particular gene, a point mutation within a gene, deletion of a whole exon, a non-coding RNA or a CNV. The genes determined as best candidates within the MCDR1 locus were chosen for screening from genomic DNA with primers designed upstream of the exons to include splice sites. *POU3F2* was identified as a possible candidate with numerous primers designed and PCR conditions attempted but all failed.

FBXL4

Yang et al. (2007) identified this gene as a possible candidate for MCDR1 but when the coding exons were screened from isolated cDNA no mutations were identified. The function of this gene in *Drosophila* highlighted it as a good candidate for both MCDR1 and PBCRA and it was selected for screening in these families (Section 8.5.1). Despite identifying SNPs within the coding exons, none were unique to affected individuals. The variations identified are shown in Table 5.7. Attempts to screen the promoter and exon 1 region failed, which was also the case for control samples. As for *POU3F2*, numerous primers were designed and PCR conditions attempted but all failed.

Table 5.7 SNPs identified in *FBXL4* in 10 MCDR1 samples.

Exon/Intron	SNP ID	Variation	Number of Affecteds with SNP
Exon 2 (5' UTR)	rs195812	A = 0.608 C = 0.392	8, also in control and PBCRA samples
Exon 5		A/G, synonynmous	1
Intron 5	rs6935315	A = 0.572 G = 0.428	4
Exon 8	rs11537982	G = 0.938 A = 0.062	2
Intron 8	rs78865887	T = 0.967 C = 0.033	1

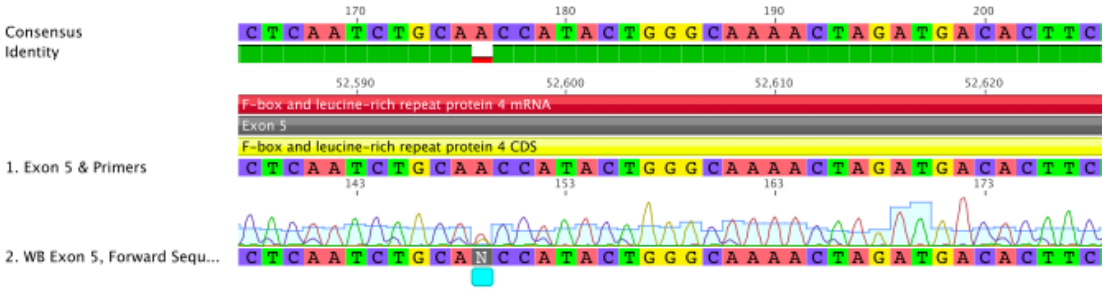


Figure 5.8 Example sequencing data showing the *FBXL4* exon 5 variation in sample WB.

SIM1

Despite this gene being downstream of the disease region refined by Yang et al. (2007), it was identified as a good candidate for PBCRA as part of this project and given the overlap in loci was included for screening in the NCMD families. The promoter and all exons were successfully sequenced including introns and the SNPs identified are summarised in Table 5.8.

Table 5.8 SNPs identified *SIM1* in MCDR1 samples.

Exon/Intron	SNP ID	Variation	Number of Affecteds with SNP
Intron 6	rs3830139	T = 0.647 G = 0.353	2
Intron 6		T/C	2
Intron 7	rs397662	T = 0.783 C = 0.217	3, also in PBCRA samples
Exon 9	rs3734354	C = 0.875 A = 0.125	1
Exon 9	rs3734355	C = 0.875 A = 0.125	1

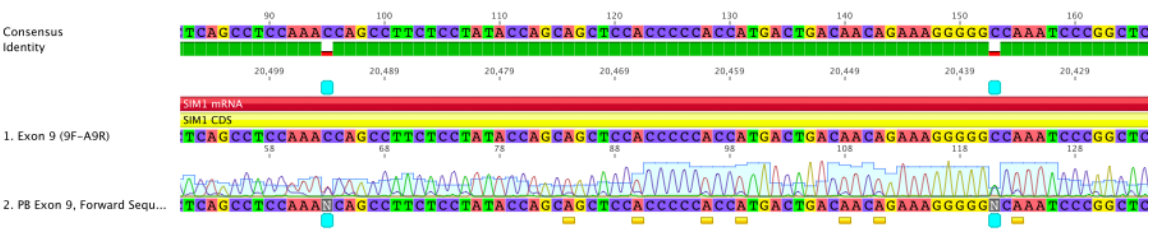


Figure 5.9 Example sequencing data showing the *SIM1* exon 9 from sample PB showing two nucleotide variations.

Within intron 8 of the *SIM1* gene is a putative novel mRNA transcript yet to be fully annotated or characterised. Despite the ORFs being incompletely identified, attempts to screen them were made. The Affymetrix 50K SNP rs3778033 (Figure 5.5) is located between the two deduced ORFs of this gene. No variations in the predicted sequences were identified.

5.5 Discussion

5.5.1 Genotyping Analysis

The analysis conducted for this disorder involved Affymetrix SNP chip analysis largely restricted to affected individuals from unrelated families. Parent-offspring data were achieved for just one family (the original British MCDR1 family) whereas from nine others just one affected member was analysed. This meant it was not possible to determine the phase of alleles and thereby derive haplotype data for the NCMD families. Despite this, comparisons were attempted between the genotype deduced from the original British samples and the genotypes of the nine other families. By comparing the genotypes of affecteds from unrelated families to the genotype of the original British MCDR1 family, it was anticipated that similarities in genotype might be identified at the MCDR1 locus that would highlight key candidate genes. Unfortunately this approach was largely unsuccessful; parent-offspring data should have been obtained for all families so that haplotypes could be compared across the disease region. Though genotype comparisons were attempted they generated very limited information as it is not known whether the apparent consistent alleles shared between all families are on the chromosome inherited from the affected parent. Another issue with the approach taken is that such differences between genotypes may occur from a comparison of any ten unrelated individuals. This

was shown to be the case when ten random HapMap genotypes at the MCDR1 locus were compared and a similar pattern of allele similarity produced. Overall therefore, determining haplotypes across all families would potentially have yielded more useful information.

Due to the size of the nine unlinked families, linkage has not been possible so the disease gene may map to a different locus such as MCDR3, which has an almost identical phenotype but links to chromosome 5p. However, MCDR3 is very rare with just a single family identified (Michaelides et al., 2003c), whereas there are numerous MCDR1 families worldwide (Section 2.5.1). Based on this it seems likely that all nine families are MCDR1 families. It is also a possibility that some families may be phenocopies, meaning they show MCDR1 characteristics but their genotypes differ with environmental conditions being responsible for the phenotype. This is a possibility for a couple of the families shown in Figure 5.3c, d & h, where an affected parent is missing.

A further issue with attempting SNP analysis rather than using traditional markers is that linkage cannot be conducted as each SNP marker has just two polymorphisms whereas microsatellites can have many more and therefore SNPs are much less informative. Many samples from large families would need to be analysed for linkage analysis to be informative using SNP technology. However, it was known that linkage would not be possible in this analysis (also due to the size of the families) and that the analysis would rely on the genotype comparisons. In hindsight it would have been useful to conduct microsatellite marker analysis with markers from previous studies used to define the MCDR1 locus as this would have indicated whether the

disease chromosome in the different families has a common origin.

5.5.2 Copy Number Analysis

The analysis carried out on the NCMD family 50K data identified a very large number of CNVs in all samples across all chromosomes. The number is in excess of those seen from other samples in this project analysed on the 250K chips, which suggests that many may be invalid. None were conserved across all MCDR1 families at the disease locus but a CN state of 3 identified in seven of the ten samples across *POU3F2* and *FBXL4* could be of interest and should be further investigated to confirm or refute the presence in all samples.

5.5.3 Candidate Gene Analysis

Though Yang et al. (2007) screened various genes within the MCDR1 locus, these genes had not been screened in the families in this project. Based on the possible roles and significance of the expressed proteins in the retina and developmental process, some were screened in this project. Splice site and promoter screening were also attempted. But for *POU3F2* and exon 1 of *FBXL4*, screening attempts failed. Numerous primers were designed using both manual and computer-generated methods (*POU3F2* shown in Figure 5.4 and Table 5.5 and *FBXL4* failed primers in Appendix I Figure 13.1 and Table 13.1) and various PCR conditions and polymerases were tried yet still the reactions failed to give the desired product. This can happen when, despite much effort to prevent it, primers can bind non-specifically. For example, *POU3F2* is not only GC rich (which makes amplification difficult as the polymerase falls away) but the gene is also similar to related members, which encourages non-specific primer binding. If further attempts were made to screen the genes/exons, it would

be worthwhile increasing the primer concentrations to encourage more to bind to the desired region. It would also be worthwhile to attempt touch-down and reverse touch-down PCR methods. The touch-down method involves using initial stringent conditions which are then relaxed as the correct product is formed to speed up the efficiency and yield. The reverse touch-down method might be more appropriate in this situation with initial PCR conditions relaxed to amplify all possible products, including non-specific regions, then gradually increasing the stringency of the reaction to encourage amplification of only the desired fragment. It may also be possible to identify restriction sites within the gene then digest the genomic DNA with specific enzymes and use the digest mixture as a template for PCR with primers designed to bind the specific overhang sites of the desired genetic region.

POU3F2 is worth screening in both these MCDR1 families and the PBCRA family because it has been shown to be involved in embryonic neuronal development and Cobrinik et al. (2006) reported that the protein product of this gene binds to promoters of retinal progenitor cells. This also makes an intriguing candidate because a binding site for this transcription factor exists on one of the promoter regions for the MCDR3 candidate *CCT5*.

In *Drosophila, dFbxl4* appears to be important for sensitivity and termination of the light receptor (Han et al., 2006). If there is a similar role of *FBXL4* in the human retina as *dFbxl4* has in *Drosophila*, then this would be a good candidate for MCDR1. It is also interesting because the related member *FBXL7*, which is listed as being expressed in the retina on the NEIBank database, was highlighted as an MCDR3 candidate (Section 6.6.1). *POU3F2* and *FBXL4* make good candidates and though Yang et al. (2007) screened them, by screening cDNAs they would not have included

splice site or promoter regions therefore it is still possible that mutations in either of these genes may still cause MCDR1. *FBXL4* was screened in MCDR1 samples and despite identifying variations in the exon sequences (including splice sites) none were unique to affected individuals.

Of the other genes in the MCDR1 locus, only *SFRS18* has been reported to be expressed in the retina and RPE/choroid. The expressed product SRrp130 has been shown to co-localise with pinin in a human corneal epithelial cell line (Zimowska et al., 2003). Though there have been no reports of SRrp130 function in the retina, given its expression pattern it may play an important role in splicing in both the retina and RPE.

Cyclin C (encoded by *CCNC*), coenzyme Q3 homolog methyltransferase (encoded by *COQ3*) and PR domain containing 13 (encoded by *PRDM13*) are less likely candidates for MCDR1. They are neither listed as being expressed in the retina or RPE/choroid nor do they have published data on roles in the eye. Ubiquitin-specific protease 45 (*USP45*) cDNA is listed in Unigene as being extracted from eye tissue and related ubiquitin-specific proteases have been identified with retina and RPE/choroid expression in NEIBank databases. Ubiquitin-specific proteases have been shown to be important in cell death in the *Drosophila* retina (Copeland et al., 2007) therefore USP45 may have an as yet unidentified role in the human retina. Interestingly, mutations in proteins involved in ubiquitination have been found to result in retinal dystrophy. For example, mutations in *KLHL7* are known to cause autosomal dominant RP (Friedman et al., 2009; Section 2.1.1). The other interesting candidate is the uncharacterised C6orf168, which may also have a role in the development or correct functioning of the retina that has yet to be determined.

Prior to this project, *MCHR2* coding exons were screened in original MCDR1 family members by Dr A. Kalhoro with no mutations found. This gene has previously been associated with appetite regulation (Hill et al., 2001) but was judged an interesting candidate because of the genes at the 6q locus there is no mouse ortholog for this gene and as mice lack a macula it was thought possible that there is a specific role for this gene in macular function.

Though outside of the locus refined by Yang et al. (2007), the genes *SIM1* and *ASCC3* should not be disregarded as candidates. Single-minded homolog 1 (encoded by *SIM1*) haploinsufficiency has been linked to obesity (Hung et al., 2007) and Bonaglia et al. (2008) have shown it is also the likely cause of obesity in a Prada-Willi-like phenotype, in which one characteristic is eye/vision abnormalities (including retinitis pigmentosa in one case). The *Drosophila sim* gene is a master regulator of neurogenesis and Holder et al. (2000) showed that mouse Sim1 is expressed in the developing kidney and central nervous system and is essential for supraoptic and paraventricular nuclei of the hypothalamus formation. Roles of SIM1 specifically in the eye have not been reported but it was screened as a candidate for PBCRA and therefore screened in the MCDR1 families as well. Variations were identified though none were in coding exons nor were variations found consistently across the MCDR1 families. They were therefore not likely to be responsible for the disease state, excluding *SIM1* mutations as the cause of MCDR1.

Though activating signal co-integrater 1 complex subunit 3 is not listed in the NEIBank retina and RPE/choroid databases, Unigene lists it as present in eye tissue and *ASCC3-like 1* is reported as being expressed in the retina and RPE/choroid. ASCC3 is of the

RNA helicase family but there are no publications on specific roles of this gene in the retina and, combined with previous linkage data that excludes this gene, this seems an unlikely candidate.

5.5.4 Summary

This analysis has highlighted that if using Affymetrix SNP chip genotyping analysis to aid in refining a disease locus, it is essential to analyse parent-offspring data and/or unaffected samples in order to determine haplotypes for comparison. The CNV analysis from the SNP chips revealed no consistent regions of CNVs in all families that might be important in the disease state. However, a CN state of 3 identified in seven of ten families may be of interest. It would appear that the primary candidates within the locus are *POU3F2, FBXL4, SFRS18, USP45* and C6orf168. Even though the coding exons of the *FBXL4* gene (including splice sites) have been screened revealing no mutations in ten NCMD families, there could still be an error in the promoter sequence of this gene, which has yet to be screened. Screening of *SIM1* provided no evidence of errors that would cause disease, suggesting the above genes highlighted that lie within the region refined by Yang et al. (2007) remain the MCDR1 candidates.

6. NORTH CAROLINA-LIKE MACULAR DYSTROPHY (MCDR3)

6.1 MCDR3 Genetic Analysis

The phenotype of this disorder is very similar to that of MCDR1 (Section 2.5.2) yet it does not link to the 6q locus and instead linkage has been found to 5p. The only phenotypic differences between MCDR1 and MCDR3 are mild abnormalities in colour vision in some of the MCDR3 affected individuals and evidence of MCDR3 disease progression, though this was only noted in a single case (Michaelides et al. 2003c). So far the pedigree depicted in Figure 6.1 is the only reported MCDR3 family in a single study publication characterising and mapping this disease. Michaelides et al. (2003c) used microsatellite markers to scan 50% of the genome and excluded linkage of MCDR3 to the MCDR1 locus in addition to other sites of the genome linked to other inherited retinal disorders (CORD6, CORD7, CORD8, *GCAP,* STGD1, STGD3 and STGD4). Significant linkage to chromosome 5p15.33-p14.3 was established and critical recombination events observed in affected member IV:9 and unaffected member V:2 at the centromeric end defined the locus as between flanking markers D5S1981 and D5S2031 (a 19.5Mb region). The markers used in the study and maximum lod scores at θ=0 are shown in Table 6.1. This is a very large locus containing many potential candidate genes and it is hoped that by generating SNP data from MCDR3 samples the disease region can be refined and the number of candidate genes reduced. It was thought that this might be possible by comparing genotype data from affected members of the family as Miyazawa et al. (2007) have shown genotyping data from such samples can be informative. As this family has already

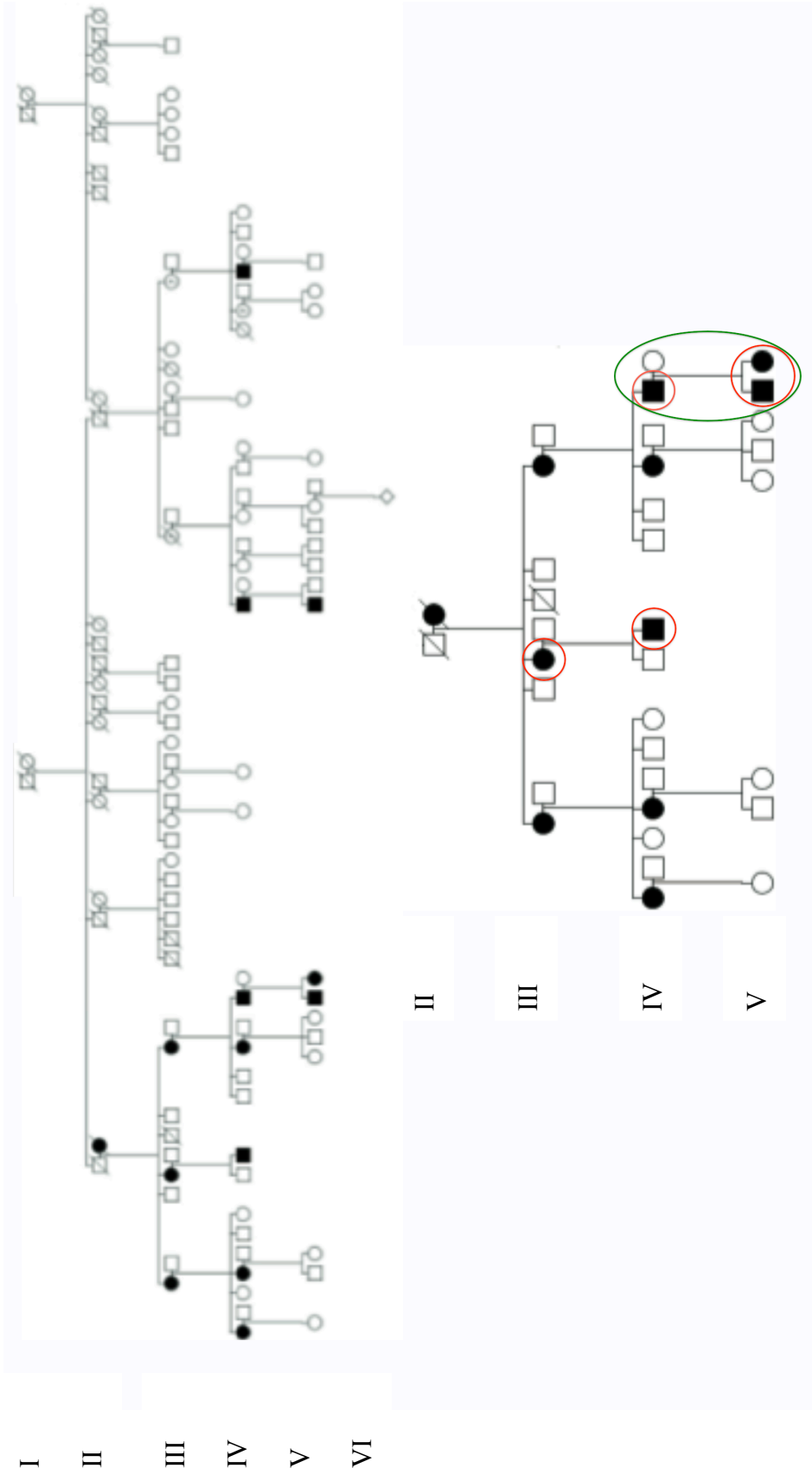


Figure 6.1 Six-generation family with autosomal dominant macular dystrophy with the branch of this family used in the study by Michaelides et al., (2003c) shown below. Circled in red are the samples run on the Affymetrix 250K *Sty* I chips and circled in green are those run on the Affymetrix 50K *Xba* I chips.

been linked the affecteds were analysed in order to compare similarities and differences in alleles between samples to help refine the locus. Though the Affymetrix Console cannot determine phase of alleles (as described in Section 4.2) and unaffected members of the MCDR3 family were not analysed, the sample cohort comprised of affected offspring plus affected parent and so the inherited disease alleles could be determined.

Table 6.1 Markers used in the study by Michaelides et al. (2003c) to determine the MCDR3 locus.

Marker	Lod Score θ = 0	Physical Position	Annotations
D5S1981	1.69	1,154,429 - 1,154,692	Within AC116351.3, genes nearby include *SLC12A7* and *TERT*
D5S417	1.93	3,121,219 - 3,121,442	Nearby genes include *IRX2* and *IRX1*
D5S2088	3.55	4,296,433 - 4,296,581	Within RP11-45O3.1, near AC026711.1. Closest genes *IRX1* and *ADAMTS16*
D5S406	3.52	4,994,064 - 4,994,359	Within CTD-2247C11.1, nearest genes *ADAMTS16* and *KIAA0947*
D5S1953	2.23	7,658,332 - 7,658,583	Within *ADCY2*, genes nearby include C5orf49, *MTRR* and *FASTKD3*
D5S630	3.61	9,561,079 - 9,561,326	Downstream of *SEMA5A* and SNORD123. *TAS2R1* nearby
D5S416	3.44	16,720,134 - 16,720,345	Within *MYO10*, genes nearby are *ZNF622* and *FAM134B*
D5S2031	- ∞	21,103,386 - 21,103,571	RP11-811J10.2

6.2 Methods Applied

6.2.1 Affymetrix Protocols

***Xba* I 50K**

Four samples, highlighted in green in Figure 6.1, were prepared for processing over the Affymetrix 50K *Xba* I chip, as described in Section 3.2.2.

***Sty* I 250K**

Five samples, highlighted in red in Figure 6.1, were prepared for processing over the Affymetrix 250K *Sty* I chip, as described in Section 3.2.3.

6.2.2 Candidate Screening

LPCAT1

Gradient BIOTaq PCRs were conducted for all primer sets (Section 3.1.1) and PCRs then carried out at the optimal annealing temperature for each primer set. The BIOTaq protocol was used and amplification products were purified and sequenced using the montage protocol unless otherwise stated (Table 6.2).

6.3 Results

6.3.1 Affymetrix Data

Genotyping Analysis

DNA from five members of the family depicted in Figure 6.1 (circled in red) were processed and run over 250K *Sty* I SNP chips. The individuals circled in green were additionally analysed on 50K *Xba I* chips. This was done as a comparison to the 250K data and although the 250K data provided more NoCall outputs than the 50K data, given the volume of SNPs that could be used for the genotyping analysis (despite abandoning those with NoCalls) it appears to have been a more useful chip. It would be valuable to process samples from extended family members in order to look at genotypes of members separated by several meioses events, which should help exclude further SNPs within the mapped region and refine the locus.

Table 6.2 Primers and annealing temperatures for *LPCAT1* exons.

Primers	Name	Annealing Temperature (°C)
CACCAGGTAGCGGAACTCCGGCTGGT ATGCGCGTGCGGGCGGCGGGA	1F 1R NEW 2	54, GoTaq with 3 minute extension
TTAGCCTTGGTCAGCGCCGGT GTCTCTGTGCTCAAGGGTGTG	2F NEW 2R NEW	61, GoTaq
TCCCTCAAGCAGATGTCCCAGC GGTCTCACTCATTCCCAACAGAGG	3F 3R	63
ACCTTCAACCCTGTCCTCTGTGAA ACCAAATGCACCTGACAGCACC	4F NEW 4R	62, GoTaq
GTGAAGATTGGCTTCTGTAAGTTCTG GACTCTGCTGCCTGCTCACTGC	5F 5R	63
GACTCTGCTGCCTGCTCACTGC ACGTGCATAGAACAGGCCGCAC	6F 6R	63
GGCTCTGGGAACCTATAAGAG CACAATGGGCTGAACCTAACGGC	7F 7R	63
CATAAGCAGGTGTTGAATCGGA CTGCAGTGAGCCCATCGCTAC	8F 8R	63
TGAAGACGGTTCTAATGGGCGT ACAGATGTGAAGTTGGCCCAG	9F 9R	63
CTGAGCCATCGGCGGTTATTCT CAGGGCAGATGTGGCAGGACA	10F 10R	64, gel-extracted
GCACCTGGCTTGACACTTGGACAG ATTGTTAGACTCCGTAATCACA	11F NEW 11R NEW	63, GoTaq extra $MgCl_2$
CTGTGGCAGCCTTGTGAACTT TCTGATGAGTGTGGATTGCT	12F 12R	63
TGCATGGCTCCACATGGAAGT TACGAACGCTGCCAGGTCTCCAG	13F 13R	64, gel-extracted
GGTACTTCAGGAAACATGCAG AAGATGTCTGCTACTGTCCGCA	14AF 14CR	60, KOD

The disorder was originally linked between flanking markers D5S1981 and D5S2031, which denotes a large disease region of over 19 million bases. It was thought that the presence of thousands of SNPs within this linked region would be able to

provide informative comparisons between the samples analysed. However, there was just a single exclude seen within the locus (running from SNPs: rs6555608, the first SNP on chromosome 5, to rs681129, position 18,105,325). In contrast, numerous excludes were seen beyond this locus (Figure 6.2), suggesting these mark the end of the disease locus, which is consistent with the previous linkage data. The exclude within the disease region was seen at rs2934551 (2,877,017), which lies near the marker D5S417. This marker was used in the original linkage study and achieved a maximum LOD score of 1.93 whilst the markers centromeric to it obtained significant LOD scores and defined the locus. As the genotyping data presented here were extracted at 0.1 confidence the single exclude within the MCDR3 locus is not likely to be a miscall, and although it is just a single marker, combined with the previous marker analysis it may suggest that the disease region lies beyond this point.

IV:14	V:7	V:8	III:4	IV:9	MCDR3 AD	dbSNP RS ID
AB	AB	AB	AB	AA	AA	rs6867384
AB	AB	AB	AB	AA	AA	rs6891956
AA	AA	AA	AA	AA	AA	rs17839277
AB	AB	AB	AB	BB	BB	rs11952141
AA	AA	AA	NoCall	AA	AA	rs1467955
AA	AA	AA	AA	AA	AA	rs643091
AA	AA	AA	AA	AA	AA	rs2652554
BB	AB	AB	AB	AA	Exclude	rs681129
BB	BB	BB	BB	BB	BB	rs17712061
AA	NoCall	AA	AB	BB	Exclude	rs2443547
AB	AB	AA	AB	NoCall	AA	rs2923765
AB	AB	BB	AB	AA	Exclude	rs2938412
AA	AB	AA	AB	BB	Exclude	rs453891
AA	AA	AA	AA	AA	AA	rs2962895
AA	AA	AB	AB	BB	Exclude	rs2950484
BB	NoCall	BB	AB	AA	Exclude	rs10461822
AA	AA	NoCall	AA	AA	AA	rs1880945
BB	BB	AB	AB	AA	Exclude	rs2962902

Figure 6.2 Excel sheet showing the group of excludes present at the centromeric end of the linked MCDR3 region. Only a single exclude output was given prior to this region.

The allele associated with the disease haplotype at rs2934551 for V:7 and V:8 is B (IV:14 is given a NoCall but given that both offspring of this individual are BB he must also have the B allele), whereas IV:9 is AA. Given that the mother of IV:9 (III:4) is AB, this suggests that a crossover may have occurred at this point in individual IV:9 (Table 6.3), indicating that the disease region lies beyond this SNP. It is difficult to be confident of a crossover from this SNP data because there are only two alleles and the Affymetrix data cannot determine which is from which parent. However, for each offspring analysed so too was an affected parent meaning the alleles inherited from the affected parent can be determined. Table 6.3 shows how the data position the possible crossover in IV:9. The purple alleles represent those that could be inherited from the affected parent. A crossover at rs2934551 would place the disease region beyond this SNP. Example data from rs2934551 (2,877,017) to rs681129 (18,105,325) are shown in Figure 6.3a and 6.3b (all SNP data from this region are shown in Appendix III).

Table 6.3 Data depicting a possible crossover event in IV:9. Purple alleles represent the disease haplotype of the affecteds.

SNP ID	MCDR3 AD	III:4	IV:9	IV:14	V:7	V:8
rs6862810	A	A A	A A	A -	A A	A A
rs4866685	B	B B	B A	B B	B B	B B
rs6890790	B	B B	B A	B B	B B	B B
rs10044519	A	A B	B A	A B	A A	A B
rs10073459	B	B A	A B	B A	B B	B B
rs2934551	X	A B	A A	B -	B B	B B
rs7710467	A	A A	A A	A A	A B	A A
rs2974647	B	B A	B A	B B	B A	B A
rs2934554	A	A B	A B	A A	A B	A B
rs4587081	B	B B	B A	B B	B A	B A

Throughout the disease region the subjects shared alleles that were of low frequency, suggesting these alleles segregated with disease. Table 6.4 identifies these SNPs, which seem to group around the sites of markers from the published study. The scattering of low frequency alleles across the linked disease region adds further evidence to the large section of chromosome 5 segregating with the disease state.

Table 6.4 A selection of SNPs at which the MCDR3 family members all exhibited a low frequency allele.

SNP ID	Physical Position	Allele Frequencies	MCDR3 Allele	Nearby Marker
rs1697976	647,816	A = 0.792 T = 0.208	T	
rs465850	2,378,836	A = 0.275 G = 0.725	A	
rs11740370	3,165,158	T = 0.908 G = 0.092	G	D6S2088
rs270629	5,077,133	A = 0.602 G = 0.398	G	D5S406
rs3776448	6,599,940	A = 0.100 G = 0.900	A	
rs326193	7,843,211	T = 0.225 C = 0.775	T	D5S1953
rs2962720	9,009,675	T = 0.608 C = 0.393	C	
rs150632	9,560,030	A = 0.108 G = 0.892	A	D5S630
rs17591495	13,536,409	T = 0.233 C = 0.767	T	
rs1440811	16,264,285	C = 0.367 G = 0.633	C	D5S416
rs11952141	18,024,800	A = 0.898 G = 0.102	G	

The 50K trio data could not be used for exclusion analysis due to the nature of the relationships between the subjects processed. This is because it is not possible to conduct two-point linkage between parents and offspring with SNP data due to the lack of

meioses and marker information (only two alleles). The three affected samples (highlighted in Figure 6.1) achieved a good QC rate of >94% but the unaffected achieved only 87%. Despite this, few NoCalls were evident at 0.1 confidence. The data were used to search for errors in Mendelian inheritance but no notable regions of error were identified.

a)

IV:14	V:7	V:8	III:4	IV:9	MCDR3 AD	dbSNP RS ID	
NoCall	BB	BB	AB	AA	Exclude	rs2934551	IRX2, C5orf38
BB	BB	AB	AB	BB	BB	rs2442698	
AB	AB	AA	AB	AB	AA	rs10866550	
AB	BB	BB	AB	AB	BB	rs11740370	CTD-2029E14.1
AB	AB	AB	AA	AB	AA	rs1661068	
AA	AA	AA	AB	AA	AA	rs4866578	RP11-121L11, AC091947.1
BB	AB	AB	AB	BB	BB	rs10042985	IRX1
AB	AB	BB	AB	AB	BB	rs2398625	
AA	AA	AA	AB	AB	AA	rs484410	CTD-2382I20.1
AB	AB	AA	AB	AB	AA	rs11134030	
AB	BB	AB	AB	AB	BB	rs7716544	RP11-445O3.1, AC026711.1
AB	BB	AB	AB	AB	BB	rs261166	
AB	AB	AB	BB	AB	BB	rs293067	RP11-445O3.2
AA	AB	AA	AB	AB	AA	rs4410622	
AB	AB	BB	AB	AB	BB	rs11746248	AC114303.1
AB	BB	BB	AB	AB	BB	rs270629	CTD-2247C11.3
AA	AB	AB	AB	AB	AA	rs13153841	ADAMTS16
AB	AA	AB	AA	AB	AA	rs2964456	
AA	AB	AA	AB	AB	AA	rs2913372	KIAA0947
AA	AA	AB	AB	AB	AA	rs17770324	
BB	AB	BB	AB	BB	BB	rs34212	
BB	AB	BB	AB	AB	BB	rs26803	
AB	AB	AB	AB	AB	AB	rs7728127	
BB	BB	AB	AB	AB	BB	rs160061	AC026797.1
AA	AB	AB	AB	AB	AA	rs1875541	
AA	AB	AB	AB	AB	AA	rs2964761	
AA	AA	AA	AB	AA	AA	rs271399	MED10
AB	AA	AB	AA	AB	AA	rs4701734	UBE2QL1
AB	AB	AB	AA	AB	AA	rs12189254	
AB	AA	AA	AB	AA	AA	rs3776448	NSUN2
AB	AB	BB	AB	AB	BB	rs10512874	SRD5A1
AA	AB	AB	AB	AA	AA	rs274667	POLS
AA	AB	AB	AB	AA	AA	rs274678	
BB	AB	BB	AB	AB	BB	rs6862164	RP11-122F24.1
AA	AB	AB	AB	AB	AA	rs4701773	
AB	AB	AA	AB	AB	AA	rs17207026	ADCY2
AB	AB	BB	AB	BB	BB	rs2287778	C5orf49

SNPs that lie within gene regions

Figure 6.3a Excel extract showing example SNP data within the MCDR3 locus.

IV:14	V:7	V:8	III:4	IV:9	MCDR3 AD	dbSNP RS ID		
AB	AB	BB	AB	BB	BB	rs2287778		C5orf49
AB	AB	AB	AB	AA	AA	rs4702506		MTRR
AA	AB	AB	AA	AA	AA	rs1503317		
AB	AB	AB	AB	AA	AA	rs1453360		RP11-334G8.1
AB	AB	AB	AB	AB	AB	rs2962124		RP11-480D4.1
AA	AA	AB	AB	AB	AA	rs12517255		
AB	AB	AB	AB	BB	BB	rs2962720		SEMA5A
AB	AB	BB	BB	AB	BB	rs899575		
BB	AB	BB	BB	AB	BB	rs13174956		
AA	AB	AB	AA	AB	AA	rs7735345		
AA	AB	AA	AB	AB	AA	rs118583		CTD-2143L24.1
AB	AB	AB	AB	BB	BB	rs4701850		
AB	BB	AB	BB	AB	BB	rs553169		
AA	AB	AB	AB	AB	AA	rs4702672		
AB	BB	BB	BB	BB	BB	rs2578619		CCT5
BB	BB	AB	BB	AB	BB	rs814586		RP11-1C1.5
BB	AB	AB	AB	BB	BB	rs34091808		ANKRD33B, DAP
BB	AB	BB	AB	AB	BB	rs1548703		
AB	AB	AB	AB	AB	AB	rs2400006		CTNND2
AA	AB	AB	AA	AA	AA	rs26148		
AA	AB	AB	AB	AB	AA	rs1900190		
BB	AB	AB	AB	AB	BB	rs4701970		
AB	AA	AA	AB	AB	AA	rs10039621		DNAH5
AB	AB	BB	AB	BB	BB	rs417750		
AB	AB	AB	AB	AB	AB	rs151473		
AB	AB	BB	AB	AB	BB	rs26114		TRIO
AB	AB	BB	AB	AB	BB	rs152639		FAM105A
AB	AB	AA	AA	AB	AA	rs1446038		
AB	AB	AB	AB	AA	AA	rs581957		FBXL7
AA	AB	AB	AB	AA	AA	rs7726523		
AB	AB	AB	AB	BB	BB	rs171817		
AB	AA	AB	AB	AA	AA	rs1867723		FAM134B
AB	AA	AA	AA	AA	AA	rs10061139		MYO10
AB	AB	AB	AB	BB	BB	rs2962360		
AB	AB	AB	BB	AB	BB	rs1398597		RP11-454P21.1
AB	AB	AB	AB	BB	BB	rs11952141		
b) BB	AB	AB	AB	AA	Exclude	rs681129		

SNPs that lie within gene regions

Figure 6.3b Excel extract showing example SNP data within the MCDR3 locus.

Copy Number Analysis

All sample data from the 250K chip were in bounds except for one (V:8), which was given an out of bounds output based on the X chromosome IQR. The analysis was run despite this and investigations of CNVs conducted.

All chromosomes were analysed for copy number variations and those common to all samples are shown in Table 6.5. Three of the five MCDR3 samples had a copy number state of 1 (indicating a heterozygous deletion at the associated SNP) within the linked

disease region at 5p15.33 but this is a known region of CNV and not seen in all samples and so is unlikely to be significant.

Table 6.5 CNVs common to all MCDR3 samples.

Chromosome	CN State	First SNP shared by all	Last SNP shared by all	Length (bp)	Published CNVs at region
5p15.33	1 (in 3 of 5 samples)	rs6862648	rs12656150	136,619	Sharp et al., (2005)
15q11.2	1	rs4114033	rs2173741	190,465	Redon et al., (2006)
18q12.1	3	rs1220029	rs9959163	165,753	

The only CNV seen in all samples but not in a region of known CNV was at 18q12.1. This would result in a gain of one copy of *CDH2*, which encodes cadherin 2, type 1 also known as N-cadherin (neuronal).

6.3.2 Candidate Gene Screening

It was hoped that the Affymetrix data would produce more exclusions and a more refined 5p locus. This was not achieved, although the region may have been reduced due to the presence of one exclude within the disease region. As the genotyping data did little to refine the locus, an extensive search of the genes within the locus was conducted to indicate which may be key candidates for screening.

There are over 60 characterised genes within the disease region and numerous other annotated genes, processed transcripts, pseudogenes and ncRNAs. Of the characterised annotated genes, the majority are listed on Unigene with their cDNA being found in eye tissue. According to the NEIBank database only two are expressed in both the retina and RPE/choroid: *SDHA* and *LPCAT1* and only this latter gene has published data providing evidence of a role in retina with Friedman et al. (2010) showing that loss of *LPCAT1* leads to photoreceptor degeneration in *rd11* mice. Cheng

et al. (2009) have also shown evidence of the importance of LPCAT1 in catalysing the inactivation of inflammatory lipids in the retina of diabetc mice.

LPCAT1

Coincidentally, *LPCAT1* was identified by another group from Montpellier, France (http://www.inmfrance.com/1_equipe1.php) as having a role in lipid metabolism that may regulate isomerase activity in the retina and based on its expression it makes a good candidate for retinal disease. This group were studying cellular and animal models for the investigation of the molecular mechanisms of regulation of the isomerisation of vitamin A. As a result of this work they discovered a new protein involved in lipid metabolism that may regulate isomerase activity and following identification of this also have evidence of *LPCAT1* expression in the retina and RPE. They hypothesised a role of the protein LPCAT1 in the regulatory mechanism of isomerase activity. This group subsequently contacted our own to suggest screening of the gene. Loss of this gene has been reported to lead to photoreceptor degeneration in *rd11* mice (Friedman et al., 2010) and the genotyping and linkage analysis for the MCDR3 family did not exclude it as a candidate. The 14 coding exons (including splice sites and sections of introns) and promoter region of this gene were screened in two selected affecteds (IV:12 and V:8) and one unaffected (V:3) from the family in Figure 6.1, with the majority of exons exhibiting no changes. The results are summarised in Table 6.6.

Table 6.6 Summary of SNPs identified in MCDR3 samples when screening *LPCAT1*.

Exon/Intron	SNP ID	Variation	Samples with SNP
Intron 1	rs1979396	A = 0.758 G = 0.242	IV:12, V:8 and V:3
Exon 2		C/T	V:3
Intron 2		G/T	IV:12 and V:3
Exon 3	rs28715640	T = 0.875 C = 0.125	V:8
Exon 3	rs3733796	G = 0.758 A = 0.242	V:8
Intron 3	rs3733797	G = 0.766 A = 0.234	V:3
Intron 3		T/G	V:8
Intron 3		T/G	IV:12, V:8 and V:3
Intron 3		G/A	V:3
Intron 8	rs27059	G = 0.792 C = 0.208	V:8
Intron 9		G/T	V:8
Intron 11	rs27056	C = 0.792 T = 0.208	V:8 and V:3
Intron 11	rs27054	C = 0.808 T = 0.192	V:8 and V:3
Intron 12	rs2277005	C = 0.583 T = 0.417	V:3
Exon 13	rs35452723	A = 0.861 G = 0.139	IV:12, V:8 and V:3
Intron 13	rs2277007	G = 0.692 A = 0.308	IV:12, V:8 and V:3

Following the identification of the non-synonymous SNP in exon 3 of V:8, which is a known SNP rs28715640 and results in a Thr125Ala substitution (Figure 6.4), other family members were screened to see if the SNP was present but it was not. Similarly, all family members were screened for the non-synonymous SNP rs3542723 in exon 13, which results in a Met427Thr missense mutation (Figure 6.5). Nine affecteds possessed the low frequency G allele but this was also seen in six of eight unaffected members, indicating it is not important to the disease state.

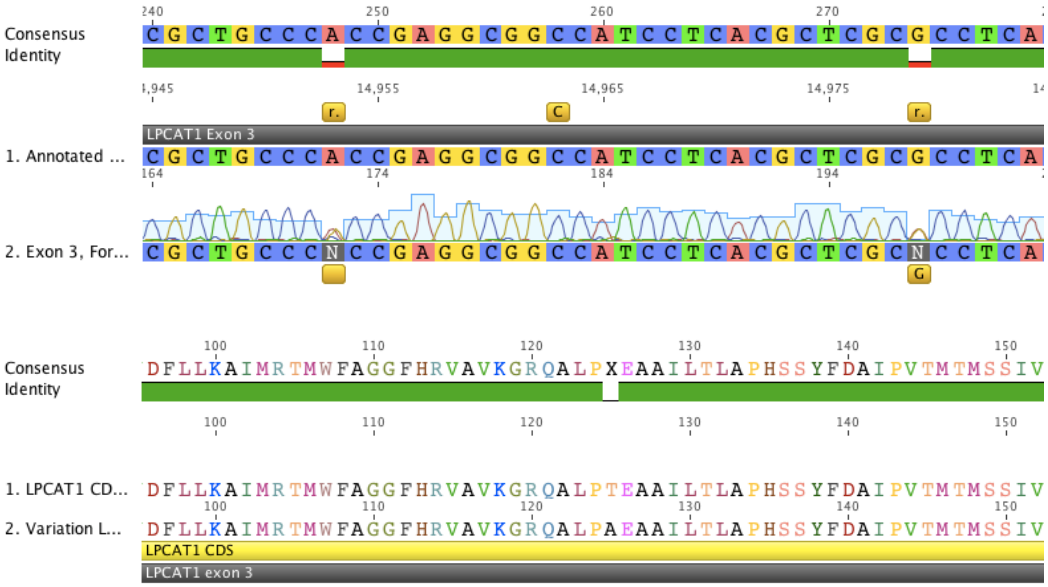


Figure 6.4 Above image shows a nucleotide trace for V:8 *LPCAT1* exon 3 with variations seen at rs28715640 (left) and rs3733796 (right). The bottom image shows the amino acid change that results from having the A allele at rs28715640 (Thr125Ala).

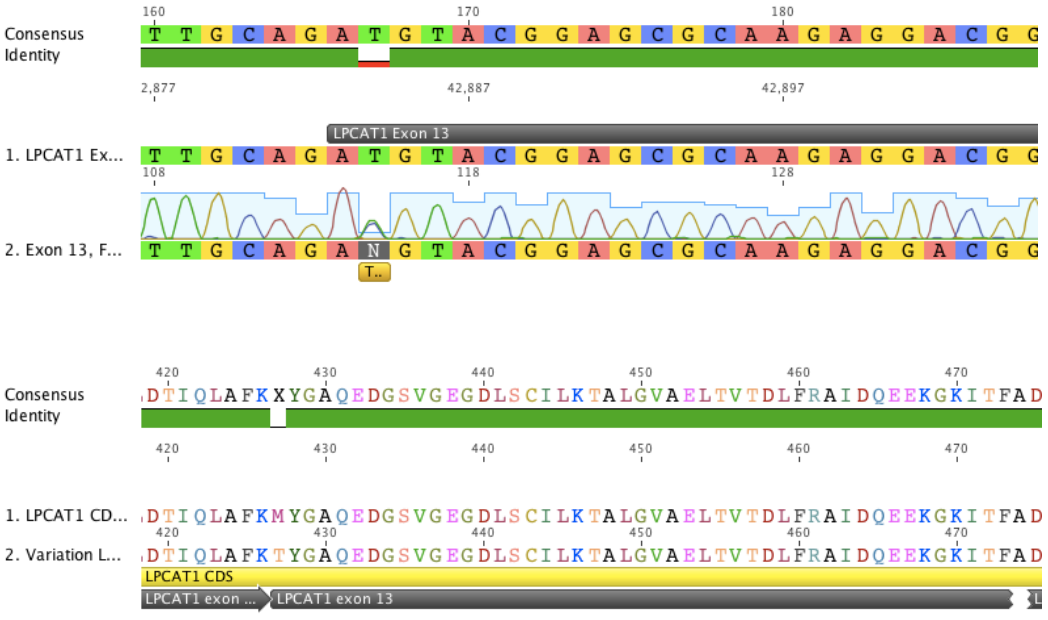


Figure 6.5 Above image shows a nucleotide trace for IV:12 *LPCAT1* exon 13 with a variation seen at rs3542723 (T/C). The bottom image shows the amino acid change that results from having the C allele (Met427Thr).

6.3.3 Bioinformatic Screening of Candidate Genes

No further genes were screened during this project but extensive analysis of further candidates was conducted. Within the original linked region are over 60 characterised genes and numerous other annotations. Of the characterised genes, a large number are listed on Unigene with their cDNA being isolated from eye tissue. Despite this, just two are listed on NEIBank as being expressed in both retina and RPE/choroid: *LPCAT1* and *SDHA*. *LPCAT1* has already been considered but *SDHA* encodes succinate dehydrogenase complex subunit A, which is part of the mitochondrial respiratory chain. Optic atrophy 1 (OPA1) and Leber hereditary optic neuropathy (LHON) are both caused by defects in mitochondrial proteins (Delettre et al., 2000; Wallace et al., 1988), which makes *SDHA* an interesting candidate.

Six of the characterised genes that lie within the original MCDR3 locus are listed on the NEIBank retina database: *CCDC127, MED10, CCT5, DAP, ANKH* and *BASP1*. *BASP1*, encoding brain abundant membrane attached signal protein 1, is a brain-soluble protein shown to be abundantly expressed in the RPE and choroid (Booji et al., 2010) and is a known promoter of neuronal growth (Mosevitsky, 2005). *MED10* encodes Mediator complex subunit 10. Whilst no specific role for this has been identified in retina, the mediator complex is known to be important for transcriptional regulation. Dürr et al. (2006) investigated whether the subunits of the mediator complex have different functions in zebrafish retinal development and suggested that the subunit composition of the complex does contribute to the control of differentiation in the vertebrate central nervous system. However, the general requirement of the mediator complex throughout the body suggests it may not be the best candidate as more systemic associations might also be expected from a fault in this complex.

Though this is not necessarily the case, as shown by mutations in splicing factor genes that cause autosomal dominant RP (Section 2.1).

CCT5 encodes chaperonin containing TCP1 (T-complex protein 1) subunit 5. This is a member of the chaperonin containing TCP1 complex, which assists in the folding of proteins and recently Posokhova et al. (2011) have shown that in mouse photoreceptors with *CCT5* suppression there is outersegment malformation and features of RP. Interestingly, regulatory transcription factor binding sites in the *CCT5* promoter region include those for *POU3F2* (a candidate for MCDR1). This could therefore be a very interesting candidate because if *CCT5* is mutated in MCDR3 and *POU3F2* is mutated in MCDR1, *POU3F2* may fail to correctly activate *CCT5* transcription. In this example, both disorders would be lacking the same protein, providing a link in genotype to match the similarity in phenotype. The nearest marker to *CCT5* used in the linkage study by Michaelides et al. (2003c) is D5S630, which achieved the highest LOD score of 3.61. It was hoped that this gene could be screened in this project but given the number of projects on the go there was found to be no time to do so. It is felt that in hindsight it would have been best to reduce the number of disorders being investigated in order to achieve more significant information on a selected two or three.

The less likely candidates from the list of six genes found in the NEIBank retina database are *DAP*, which encodes death-associated protein and acts as a positive mediator of programmed cell death, and *ANKH*. This latter gene encodes ankylosis progressive homolog (mouse) and no retina-specific functions have been identified for either.

A further five of the genes in the MCDR3 locus are listed in the NEIBank RPE/choroid database: *PCDC6, KIAA0947, ADCY2 FAM105B* and *FBXL7*. This latter gene is an interesting candidate because the related member, *FBXL4,* is a candidate for MCDR1 and given the similarities between the two disorders, mutations in genes from the same family could cause the diseases and explain the similarities in phenotype. Whilst expression of *FBXL7* has been found in the RPE/choroid, there is no published data on particular roles of this gene in the eye. *FAM105B* encodes family with sequence similarity 105, member B but little is known about the protein. Though there is no specific information on the protein product, a protein family database (PFAM, Finn et al., 2010) sequence search reveals a highly conserved peptidase C65 domain that is specific to ubiquitin isopeptidases. Despite the lack of information on this gene it cannot be ignored as a candidate. *ADCY2* also makes a less likely candidate but not one to be excluded. It encodes adenylate cyclase type 2 (brain), a membrane-associated enzyme that is Ca^{2+}/calmodulin insensitive and catalyses the formation of the secondary messenger cyclic adenosine monophosphate (cAMP). Nicol et al. (2006) investigated the roles of adenylate cyclases in the development of retinal projections and identified Adcy2 in the developing retinal ganglion cell layer in the barrelless mouse strain. Based on this evidence, this gene may have a role in the normal development of the human retina.

Other genes that lie within the 5p15.33-p14.3 region that are not listed on NEIBank databases yet have been shown to be important in the retina are: *IRX4* (Jin et al., 2003); *TERT* (Lau et al., 2007); *SEMA5A* (Goldberg et al., 2004) and *TRIO* (Newsome et al., 2000). *IRX4* was shown by Jin et al. (2003) to regulate *Slit1,* the product of which guides the growth of retinal axons and research in

Drosophila also identified a role for Trio in photoreceptor axon extension (Newsome et al., 2000). Telomerase reverse transcriptase (TERT) was suggested by Lau et al. (2007) to have a role other than telomere maintenance in the vertebrate central nervous system. Cloning and characterisation of TERT in zebrafish also provided evidence that TERT activity in the retina has other functions (Lau et al., 2008). *SEMA5A* makes an interesting candidate not only because it lies downstream of the high-scoring marker D5S630 from the original mapping study, but also because it has been shown to inhibit axon growth in retinal ganglion cells (Goldberg et al., 2004) and helps ensheath retinal axons during optic nerve development (Oster et al., 2005). Interestingly, mutations in the related member *SEMA4A* have been shown to cause retinitis pigmentosa and cone-rod dystrophy (Abid et al., 2006).

Of these candidates, *CCDC127, SDHA, PCDC6, TERT, LPCAT1* and *IRX4* lie prior to the single exclude generated in the MCDR3 AD genotyping analysis. *KIAA0947, MED10, ADCY2, SEMA5A, CCT5, DAP, TRIO, FAM105B, ANKH, FBXL7* and *BASP1* occur after the exclude. So, if including the whole original disease region, primary candidates are suggested to be: *SDHA, CCT5, BASP1* and *SEMA5A*. *LPCAT1* was screened in this project but no disease-causing mutations were identified. The genotyping data suggests the disease region lies after rs2934551, and so the primary candidates are suggested to be: *CCT5, SEMA5A, BASP1, TRIO* and *FBXL7*.

6.4 Discussion

6.4.1 Genotyping Analysis

The 250K genotyping analysis confirms the previous mapping data that linked MCDR3 to 5p15.33-p14.3 between flanking

markers D5S1981 and D5S2031 (Michaelides et al., 2003c). From the first SNP on chromosome 5 (at 5p15.33) down to rs681129 (18,105,325) there was just a single excluded SNP. In contrast, numerous excludes were seen beyond this point (Figure 6.2) and across the rest of chromosome 5. This suggests a large region between 5p15.33-p14.3 segregates with disease, including a number of low frequency alleles (Table 6.4) and the Affymetrix genotyping analysis has been able to refine the disease locus. Linkage analysis was not conducted as it would be uninformative with such data because normally, for significance to be achieved, at least ten meioses must exist between samples which does not occur in the data set used here (Figure 6.1). In addition to this, SNP data are not overly informative, relying on just two alleles, whereas traditional markers can have numerous genotypes.

Unlike with the MCDR1 genotyping analysis, the problem of determining phase was not an issue here because affected parents of affected offspring were analysed, enabling the determination of the inherited disease allele. The alleles were compared amongst all affecteds to identify those consistent in all affecteds. Where there were alleles that were inconsistent, the SNPs could be excluded as affecteds from the same family would be expected to carry the same haplotype at the disease locus. As it turned out the Affymetrix genotying data was not as informative as had been hoped as only a single excluded SNP was present within the disease region at rs2934551 (2,877,017). As this was excluded from data extracted at 0.1 confidence it is unlikely to be a miscall. It is possible that this exclude is indicative of a crossover in individual IV:9 (Table 6.3). This excluded SNP also lies near the marker D5S417, which was used in the original linkage study and achieved a maximum LOD score of 1.93, whilst markers centromeric to it obtained significant scores. As significant linkage

was only seen after rs2934551 this may also be evidence that the disease region lies beyond this excluded SNP. If this is the case then it refines the locus and reduces the number of candidate genes.

It was expected that comparison of the 250K genotyping data would provide many more excludes and further refine the disease locus. Indeed when looking at similar samples from a single MCDR4 family, numerous excludes were made across all chromosomes and the linked disease locus, but this did not occur here. As the genotyping data did little to refine the MCDR3 locus, an extensive search of the genes within the locus was conducted to identify the best candidates.

6.4.2 Bioinformatic Searching for Candidate Genes

As the screening of many candidate genes was outside the remit of this project, extensive searching of the candidates within the disease region was conducted in order to identify the primary candidates for future screening projects. The bioinformatic screening identified *CCT5, SEMA5A, BASP1, TRIO* and *FBXL7* as the primary MCDR3 candidates.

6.4.3 Copy Number Analysis

All chromosomes were analysed for CNVs in all samples and those seen consistently in all samples were identified. Though a CNV was identified in the disease locus at 5p15.33, this was not common to all samples and resided in a known CNV spot, therefore making it unlikely to be important in the disease state.

Just two regions were identified where all samples shared a CNV, these were at 15q11.2 at a known region of CNV and at 18q12.1. This latter region is not a known region of CNV and would result

in an extra copy of *CDH2*. N-cadherin has been found in retinoblastoma and normal neural tissue (van Aken et al., 2002). It has been shown to be important in the patterning of *Drosophila* retina through cellular surface mechanisms (Hayashi and Carthew, 2004). Though this gene may play a similar role in the human retina, having an extra copy is unlikely to be significant in the MCDR3 phenotype given that the disorder links to 5p.

6.4.4 Summary

The Affymetrix analysis suggests that MCDR3 is not caused by a copy number variation at the chromosome 5 locus. The genotyping analysis appears to have refined the MCDR3 locus to a 16.5Mb region and extensive bioinformatic analysis has been conducted to identify the best candidates within the locus. The disease does not seem to be caused by mutations in *LPCAT1*. The primary candidate is suggested to be *CCT5*.

7. NORTH CAROLINA-LIKE MACULAR DYSTROPHY AND PROGRESSIVE SENSORINEURAL HEARING LOSS (MCDR4)

7.1 MCDR4 Genetic Analysis

This disorder has a very similar ocular phenotype to MCDR1 but does not link to 6q and has an additional feature of progressive sensorineural hearing loss. The phenotype, outlined in Section 2.5.3, has only been described in a single family (Francis et al., 2003, Figure 7.1). As for MCDR3, 50% of the genome was screened in members of this family using microsatellite markers and candidate regions mapped to other inherited retinal diseases were also excluded in the analysis, including: *ABCA4*, STGD4, CORD7, *GCAP*, *ELOVL4* and *TIMP3*.

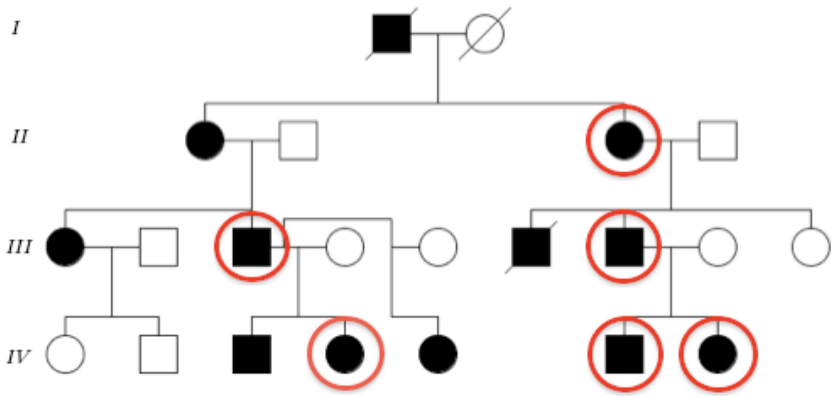


Figure 7.1 Four-generation pedigree of a family with autosomal dominant macular dystrophy and progressive sensorineural hearing loss. Red circles indicate patient samples run on the Affymetrix 250K *Sty* I chips.

Unfortunately with the family depicted in Figure 7.1 being the only one currently known to have this disorder, it was difficult to obtain statistically significant linkage data. However, following screening of around 50% of the genome, a maximum positive LOD score of 2.92 was obtained with the marker D14S261 (14q11.2), Table 7.1. Given the small family, this is likely to be as significant a LOD score as could be achieved and therefore indicates the disease locus. The authors of the study conducted a search of online databases of genes within the linked region but at the time found none to be expressed in both the retina and cochlear.

In this project the aim is to refine the 14q disease locus in this family and identify candidate genes or possible disease-causing CNVs. As with the MCDR1 and MCDR3 investigations, the approach to refining the disease-causing region was based on microarray SNP analysis. As for the MCDR3 analysis, it was felt that by comparing genotypes of affected individuals across the disease locus, SNPs where there was no consistent allele could be excluded and therefore refine the disease region by highlighting regions of shared genotypes. By running the affected parent of affected offspring the disease haplotype could be determined.

Table 7.1 MCDR4 markers used to link the disease to chromosome 14.

Marker	Lod Score $\theta = 0$	Physical Position	Annotations
D14S261	2.92	20,840,502 – 20,840,692	Within *TEP1*, nearby genes include *CCNB1IP1*, *PARP2*, *OSGEP* and *TMEM55B*
D14S72	$-\infty$	21,371,011 – 21,371,277	Nearby genes include *RNASE3* and *FAM12B*, *RPGRIP1* downstream
D14S1023	$-\infty$	21,442,084 – 21,442,186	Nearby genes include *RNASE2* and *MET11D2*, *RPGRIP1* downstream
D14S283	0.54	22,687,545 – 22,687,675	Within *TRAC*

7.2 Methods Applied

7.2.1 Affymetrix 250K Protocol

Six samples, highlighted in red in Figure 7.1, were prepared for processing over the Affymetrix 250K *Sty* I chip, as described in Section 3.2.3.

7.2.2 Candidate Screening

NOVA1

Gradient GoTaq PCRs were conducted for all primer sets (Section 3.1) and PCRs were then carried out at the optimal annealing temperature for each primer set. The GoTaq protocol (Section 3.1.2) was used and amplification products were gel-extracted and sequenced (Table 7.2). Exon 6 failed to amplify despite attempts with GoTaq, Extensor and BIOXACT Long protocols (Section 3.1, primers shown in Appendix IV).

Table 7.2 Primers and annealing temperatures for *NOVA1* exons.

Primers	Name	Annealing Temperature (°C)
GAGAAATAATGAGCGAGACCG	1F	62
TACATGGTCAACCTCTGGACCGAT	NEW 1R	
CCTGCAGTTATATACCCAGTGC	2F	57
TGGTCTAATTCACATACACGGT	2R	
CTTCTCTGGATCTCTGATTGAGC	3F	55
ATGAGACCTTGATAGTCTTTA	3R	
TCATGAATATGGTCAGGATACT	4F	56
TGGAATCTGACTATGGTGCAT	4R	
TATTCTGTCCACTATACTCTGT	5F	56
CGGTATGTACTGAAACATGTC	5R	

7.3 Results

7.3.1 Affymetrix Data

Genotyping Analysis

All samples achieved a QC of >96% and genotypes were extracted at 0.1 confidence. The MCDR4 AD genotypes were generated for chromosome 14 and whereas MCDR3 AD genotypes for the chromosome 5p locus produced just one exclude within the disease region, across chromosome 14 (and indeed the other chromosomes, examples in Appendix V), the MCDR4 AD produced many exclude outputs. This indicated inconsistent alleles were present between affected individuals of the same family and so could be excluded from the disease locus as they would be expected to have the same haplotype at the disease interval. This is what was hoped would be achieved before beginning the analysis but was not seen across the large MCDR3 locus. Between excludes on chromosome 14, the longest run of contiguous SNPs with shared alleles overlapped with the marker D14S261 and may define the MCDR4 locus (Figure 7.2).

The region runs between excludes at rs11623837 (20,762,066) and rs4981261 (21,060,447) and contains the genes: *TTC5, PARP2, TEP1, CCNB1IP1, KLHL33, OSGEP, APEX1, TMEM55B, PNP* and *RNASE10, 9, 11* and *12*; the ncRNAs RNaseP_nuc, 5SrRNA, SNORA79 and SNORD126; and the protein coding annotations AL355075.1 and AL163195.1 (shown in Figure 7.2). Given that the disorder has been linked to 14q with the marker D14S261, these data refine the region around the marker and suggest the locus is in the ~300kb region shown in Figure 7.2. SNPs near the marker D14S1023 were excluded (Figure 7.3), which correlates with the marker data from the original study.

MCDR4 AD	dbSNP RS ID			MCDR4 AD	dbSNP RS ID		
Exclude	rs10134743		TTC5	AA	rs17211432		KLHL33
Exclude	rs11623837			AA	rs6575879		AL355075.1
AA	rs2318864		CCNB1IP1	AA	rs8010845		
AA	rs10873395		SNORA79, SNORD126	BB	rs1878705		
AA	rs7153417		RNaseP_nuc	AA	rs11160711		OSGEP
BB	rs3093872		RNaseP_nuc, PARP2	BB	rs938881		
BB	rs4981158		PARP2	BB	rs999692		
BB	rs7161611		TEP1	BB	rs883037		
BB	rs1713448		D14S261	AA	rs3136818		APEX1
AA	rs1760907			BB	rs1065749		TMEM55B, PNP
AA	rs4982038			AA	rs11850059		
BB	rs1713417			AA	rs11850069		
AA	rs938892			BB	rs17242662		
AA	rs11160462			BB	rs12147450		
BB	rs17111211			AA	rs17308401		
BB	rs2275009			AA	rs17112431		
AA	rs1713435		5S_rRNA	AA	rs12891826		
AA	rs1760955			BB	rs7161172		
AA	rs1953226			AA	rs2840281		RNASE10
AA	rs12436843			AA	rs11628127		
BB	rs12587478		KLHL33	BB	rs1243705		
BB	rs11160645			BB	rs7159719		
AA	rs17211432			BB	rs1243704		
AA	rs6575879		AL355075.1	AA	rs7148897		
AA	rs8010845			BB	rs17242692		
BB	rs1878705			AA	rs1243698		
AA	rs11160711		OSGEP	BB	rs1243648		RNASE9
BB	rs938881			AA	rs6576288		RNASE11, RNASE12
BB	rs999692			BB	rs10151120		RNASE11, AL163195.1
BB	rs883037			AA	rs4981260		
AA	rs3136818		APEX1	Exclude	rs4981261		
BB	rs1065749		TMEM55B	Exclude	rs4981265		

SNPs that lie within gene regions

SNPs closest to original markers

Figure 7.2 Excel sheet extract showing the longest run of contiguous shared alleles at 14q11.2 between MCDR4 affecteds, this overlaps with the original high-scoring marker D14S261.

III:3	IV:4	II:3	III:7	IV:6	IV:7	MCDR4 AD	dbSNP RS ID		
AA	AA	BB	AB	NoCall	AB	Exclude	rs1243446		D14S1023, NDRG2
AB	AA	NoCall	AB	AB	AB	AA	rs1243450		
AB	BB	AA	AA	AA	AA	Exclude	rs1243459		TPPP2
BB	BB	AA	AB	AB	AB	Exclude	rs2282035		TPPP2, RNASE13

Figure 7.3 Excel sheet extract showing exclude sites around the D14S1023.

The gene *RPGRIP1* (RP GTPase regulator interacting protein 1), mutations in which have been found to cause recessive LCA (Dryja et al., 2001) and recessive cone-rod dystrophy (Hameed et al., 2003), lies at the 14q11.2 locus and the region around this gene was not excluded by the MCDR4 AD analysis (Figure 7.4). This gene lies between excludes at rs10483251 (21,671,277) and rs10135215 (21,947,857). In this region are the annotations:

AL157687, *HNRNPC, RPGRIP1, SUPT16H, CHD8,* two snoU16-53 and RP11-689J19.1. The marker D4S283 lies within excluded SNPs close to one another but is not actually excluded itself, which again seems consistent with previous marker analysis (data not shown).

Also at 14q11.2 is *NRL*, mutations in which cause autosomal dominant RP (Bessant et al., 1999). This region appeared to be excluded by the MCDR4 AD, Figure 7.5.

III:3	IV:4	II:3	III:7	IV:6	IV:7	MCDR4 AD	dbSNP RS ID		
AA	AA	BB	AB	AA	AA	Exclude	rs10140795		
AA	AA	BB	AB	AB	AB	Exclude	rs2319685		
BB	AB	AA	AB	AB	AB	Exclude	rs10483251		
BB	BB	BB	BB	BB	BB	BB	rs17102536		AL157687
AA	AA	AA	AA	AA	AA	AA	rs17102539		HNRNPC
BB	BB	AB	BB	NoCall	AB	BB	rs8009894		
AA	AA	AA	AA	NoCall	NoCall	AA	rs4982437		
AA	AA	AB	AA	AB	AB	AA	rs7141230		
BB	BB	BB	BB	AB	AB	BB	rs17092523		
BB	BB	BB	BB	BB	BB	BB	rs8021748		RPGRIP1
AA	AA	AA	AA	AA	AA	AA	rs7157052		
AA	AA	AA	AA	AA	AA	AA	rs10872898		
AA	AA	AA	AA	AA	AA	AA	rs10782384		SUPT16H
AA	AA	AA	AA	AA	AA	AA	rs10132333		
BB	BB	BB	BB	BB	BB	BB	rs7152833		CHD8, snoU6-53
AA	AA	AA	AA	AA	AA	AA	rs17792659		CHD8, RP11-689J19.1
AA	AA	BB	BB	AB	AB	Exclude	rs10135215		RAB2B, TOX4
AB	AB	AA	AB	BB	BB	Exclude	rs11626693		METTL3
AA	AA	AA	NoCall	BB	BB	Exclude	rs17792778		SALL2
AA	AB	AB	AB	AB	AB	AA	rs2319872		OR10G3
NoCall	BB	AB	AA	AB	AB	Exclude	rs8011308		OR10G2

Figure 7.4 The MCDR4 AD does not exclude the genomic region for *RPGRIP1*.

III:3	IV:4	II:3	III:7	IV:6	IV:7	MCDR4 AD	dbSNP RS ID		
AB	BB	AB	NoCall	AA	AA	Exclude	rs1951634		NRL, PCK2
AB	AA	AB	AA	AB	AB	AA	rs6573565		DCAF11
AB	BB	AB	NoCall	AA	AA	Exclude	rs927494		
AB	AA	AB	AA	AB	AB	AA	rs11574503		FITM1
AB	BB	AB	AB	AA	AA	Exclude	rs3742500		FAM158A, PSME2

Figure 7.5 Excel extract showing exclusions around the genomic region for *NRL*.

Another long run of contiguous SNPs at 14q in which all samples shared alleles was between excludes at rs2332676 (26,654,330) and rs12435522 (27,179,913). This region contains only *NOVA1*

and the protein coding annotations AL132716.1 and AL132716.2, shown in Figure 7.6.

III:3	IV:4	II:3	III:7	IV:6	IV:7	MCDR4 AD	dbSNP RS ID		
AA	AA	AA	AB	AB	AB	AA	rs10498286		
NoCall	AA	AA	NoCall	AB	NoCall	AA	rs17496181		NOVA1
AA	AB	AB	AA	AA	AA	AA	rs7154811		AL132716
AA	NoCall	NoCall	NoCall	AA	AA	AA	rs178169		
AA	AB	AB	AA	NoCall	AB	AA	rs17111388		
AA	AA	AA	AA	AA	AA	AA	rs4983176		
AA	AA	AA	AA	AA	AA	AA	rs1955819		

Figure 7.6 Excel image showing the region for *NOVA1* is not excluded by the MCDR4 AD.

There were few SNPs at which individual genotypes were heterozygous and therefore looking for rare alleles that segregate with the disease state was difficult. Two were identified near the marker D14S261, rs1243684 (A=0.153, G=0.847) and rs1087395 (T=0.275, C=0.725), Table 7.3.

Table 7.3 A selection of SNPs at which the MCDR4 family members exhibited a low frequency allele.

SNP ID	Physical Position	Allele Frequencies	MCDR4 Allele	Marker/Genes Nearby
rs1087395	20,775,845	T = 0.275 C = 0.725	T	D14S261
rs1243684	20,891,598	A = 0.153 G = 0.847	A	D14S261
rs1878705	20,905,515	T = 0.625 C = 0.375	C	
rs999692	20,917,665	A = 0.675 G = 0.325	G	*TMEM55B*
rs7141230	21,718,739	T = 0.367 C = 0.633	T	*RPGRIP1*
rs2319872	22,048,577	C = 0.525 T = 0.475	T	
rs4982689	23,321,359	C = 0.308 G = 0.692	C	
rs3759611	23,836,212	T = 0.850 C = 0.150	C	
rs6573565	24,594,733	A = 0.308 G = 0.692	A	*NRL*
rs7154811	26,996,510	T = 0.225 C = 0.775	T	*NOVA1*

Copy Number Analysis

The copy number analysis data quality were determined to be in-bounds for all samples and all chromosomes were analysed for structure variations. All CNVs were checked against the DGV to eliminate those that were detected in regions of known CNV. There were few examples of CNVs being shared in all affecteds and all were in known regions of CNV. These are shown in Table 7.4.

Table 7.4 CNVs to all MCDR4 samples.

Chromosome	CN State	First SNP shared by all	Last SNP shared by all	Length (bp)	Published CNVs at region
4q28.3	3	rs12647096	rs17513517	196,848	Redon et al., (2006), Korbel et al., (2007), Pinto et al., (2007), Perry et al., (2008)
5p11	3	rs6451824	rs4866974	47,034	Korbel et al., (2007), Perry et al., (2008b), Itsara et al., (2009)
14q11.2	1 (in all but one sample)	rs17197802	rs11157508	180,226	*TRAC* region known to vary structurally

7.3.2 Candidate Screening

NOVA1

This gene was chosen to be screened due to its expression in the cochlear and potential role in neurological pathways. The coding exons were screened from genomic DNA but attempts to amplify exon 6 failed (this occurred in control DNA samples as well). GoTaq gradients, Extensor Mix and BIOXACT Long PCR methods were used in addition to splitting the exon 6 sequence into three small amplicons but all failed to amplifiy. No variations were identified in the other exons of samples screened. There appear to be different splice variants of *NOVA1*, in one variant of the gene, exons 1, 2, 3, 4 and 6 encode the product, whereas another uses exons 1, 2, 3 4 and 5. For the first variant, exon 6 provides 50% of the coding sequence, so this exon needs to be fully screened before *NOVA1* can be excluded as a candidate.

7.4 Discussion

7.4.1 Genotyping Analysis

Analysing affected parents and their affected offspring of the MCDR4 family in Figure 7.1 allowed for identification of the disease alleles across the 14q locus. Runs of alleles shared between all affecteds would be expected in members of the same family at the disease locus. Where the disease allele was not consistent the SNPs could be excluded with long runs of consecutive shared alleles indicative of a shared haplotype and possible disease locus. Whereas with the MCDR3 analysis these allele comparisons did little to refine the disease locus, a similar sample cohort from the MCDR4 family provided many exclusion areas. These were seen across all chromosomes, including chromosome 14. The AD genotypes for chromosome 14 produced numerous exclude outputs across the whole chromosome. This indicated differences between the alleles of samples and the longer runs of shared alleles between SNPs would more likely represent the disease locus. Between excludes, the largest run of SNPs at which all samples shared a common allele overlapped with the marker D14S261 (Figure 7.2) and covered around 300kb.

Other genes associated with retinal disease have been linked to 14q11.2: *NRL* and *RPGRIP1*. *NRL* (neural retina leucine zipper) appears to be excluded as a candidate by the MCDR4 AD analysis as the affected members carry different alleles over this region. In contrast, *RPGRIP1* was not excluded by the genotype comparison analysis. Mutations in retinits pigmentosa GTPase regulator interacting protein 1 (RPGRIP1) were identified in LCA patients (Dryja et al., 2001) and CORD families (Hameed et al., 2003). Though the AD analysis does not exclude the *RPGRIP1* region on 14q11.2, this gene is considered to be tissue-specific and only

expressed in the rod and cone photoreceptors of the retina. MCDR4 has an NCMD phenotype with associated sensorineural progressive hearing loss. It would therefore be expected that the gene responsible for the disorder will be expressed in both retinal and cochlear cells, suggesting *RPGRIP1* is not a likely candidate.

7.4.2 Bioinformatic Searching for Candidate Genes

Within the run of SNPs overlapping the marker D14S261 are thirteen characterised genes, two protein coding annotations and four ncRNAs. Of the genes, *CCNB1IP1* and *TEP1* are listed on Unigene as having their cDNA extracted from both eye and ear tissue. *TTC5, PARP2, KLHL33, OSGEP, APEX1, TMEM55B* and *PNP* cDNAs were found in eye tissue (ear samples not tested). In both the NEIBank cochlear and RPE/choroid databases is *CCNB1IP1*, whilst *OSGEP* is listed in the retina and RPE/choroid databases and *TMEM55B* in the retina (with the related *TMEM55A* in cochlear) database. Other genes highlighted at 14q11.2 but not in the region around D14S261 include: *HNRNPC*, which is near *RPGRIP1* and listed as being expressed in the RPE/choroid, retina and cochlear and *NOVA1*, which NEIBank lists only in the cochlear database. Though *NOVA1* (neuro-oncological ventral antigen 1) has no listed expression in the retina, Unigene lists cDNA of *NOVA1* as being isolated from eye and, in particular, ear tissue. It is expressed in the central nervous system and is associated with the autoimmune disease paraneoplastic opsoclonusmyoclonus ataxia (POMA) (Buckanovich et al., 1993). Though no role for this gene has been specifically associated with retinal or cochlear function, given its expression pattern and that it is expressed in neurons it is plausible to consider that a mutation in this gene may cause problems in neurons of the retina and in the ear. The coding exons of this gene were screened in members of the MCDR4 family but no variations were

identified. Unfortunately there was incomplete screening of exon 1 and attempts to screen exon 6 failed so this gene cannot be excluded as a candidate. Numerous attempts were made to screen these exons with various primers designed, different reaction conditions and reagents used yet the attempts failed. Future techniques could be tried as described in Section 5.5.3.

Good candidates that lie within the long run of contiguous SNPs across D14S261 at which the MCDR4 samples share common alleles, include *CCNB1IP1, OSGEP* and *TMEM55B*. There is no published data on cyclin b1-interacting protein (CCNB1IP1) roles in the retina but cyclin b1 involvement in retinal development has been reported (Barton and Levine, 2008). *OSGEP* is an unlikely candidate as it encodes O-sialoglycoprotein endopeptidase, with a general function that is not tissue-specific. *TMEM55B* is a more interesting candidate as it encodes transmembrane protein 55B, expression of which was detected in the retina with the related member *TMEM55A* expression detected in the cochlear. Recently *TMEM216* mutations have been shown to cause ciliopathies (Valente et al., 2010). Though the roles of TMEM55B are unknown, expression of the related members in the retina and ear are intriguing because the sensory cells of both have cilia structures. If like *TMEM216*, *TMEM55B* is expressed in the ciliary structures, it is possible a mutation may also cause a ciliopathy and therefore a retinal and cochlear phenotype.

Other genes highlighted at 14q11.2 by the AD genotyping analysis that are listed in Unigene as being detected in eye tissue include: *TEP1, TTC5, PARP2, KLHL33, APEX1* and *PNP. TEP1* is an interesting candidate as it encodes telomerase associated protein 1 and its activity is mediated by TERT, the gene for which was identified as a candidate for MCDR3 (Section 6.6.3). *TTC5* encodes

tetratricopeptide repeat domain 5 and though there is little information on the roles of this product, *AIPL1* (mutations in which cause LCA and CORD, Sohocki et al., 2000) was shown by Hidalgo-de-Quintana et al. (2008) to have a tetratricopeptide domain, mutations in which compromised the interactions with chaperones. Based on this it seems possible that TTC5 has a role in forming chaperone complexes for proteins important in the retina/ear. Interestingly, the MCDR3 candidate *CCT5* has a chaperonin function and contains a transcription factor site for *POU3F2* binding (a candidate for MCDR1). Given the similarities in phenotypes of MCDR1, MCDR3 and MCDR4, genes whose functions link together like this are of great interest and make intriguing candidates. The MCDR4 candidate *PARP2* also has a promoter region for binding *POU3F2* but encodes poly(ADP-ribose) polymerase 2, which seems an unlikely candidate for MCDR4 given the general function of the protein. This is also the case for *PNP,* which encodes purine nucleotide phosphorylase.

The roles of kelch-like 33 (Drosophila), encoded by *KLHL33*, are unknown which makes it difficult to consider as a candidate. However, mutations in kelch-like 7, a member of the BTB (broad-complex, tramtrack, bric-a-brac) kelch superfamily, have been found to result in autosomal dominant RP (Friedman et al., 2009). Finally, apurinic endonuclease (APEX1) seems a better candidate as it appears to be associated with differentiation in the retina. Chiarini et al. (2000) studied expression in the retina of developing rats and found levels of APEX1 appeared to increase in differentiating cells but in apoptotic cells it was absent.

From the chromosome 14 Affymetrix AD genotyping analysis of the MCDR4 family members, the main candidates are suggested to be: *TEP1, TTC5, TMEM55B, CCNB1IP1* and *NOVA1*.

7.4.3 Copy Number Analysis

The CNV analysis of all chromosomes in all samples revealed few regions where all samples shared a common CNV. A CNV was identified in five of six samples at the disease linked 14q11.2 but as this occurred at the polymorphic *TRAC* (T cell receptor alpha constant) region and not in all samples it is unlikely o be disease-associated. The only other two CNVs shared by all samples were at 4q28.3 and 5p11 and these were at known regions of CNV.

7.4.4 Summary

The 14q11.2 locus was investigated in the MCDR4 family and detailed genotyping analysis conducted. This analysis excluded *NRL* but not *RPGRIP1*, however, the photoreceptor-specific expression of this latter gene does not make it a likely candidate due to the associated hearing phenotype of this disorder. *NOVA1* was identified as an interesting candidate but screening of exons 2 to 5 of this gene revealed no mutations (promoter, exon 1 and 6 screening needs to be repeated). The longest run of contiguous SNPs with shared alleles between MCDR4 affecteds highlighted *TTC5, TEP1, CCNB1IP1* and *TMEM55B* as good candidates.

8. PROGRESSIVE BIFOCAL CHORIORETINAL ATROPHY (PBCRA)

8.1 Genetic Analysis

Progressive bifocal chorioretinal atrophy was identified in a large family over 50 years ago and is an interesting disorder as it links to an overlapping region on 6q with the MCDR1 locus yet has a distinct phenotype. If the same gene is involved in both disorders it seems likely that different mutations would be identified. The linkage analysis conducted by Kelsell et al. (1995) on the family in Figure 8.1 demonstrated significant linkage with seven 6q marker loci and due to recombination events in unaffected III:16 between D6S249 and D6S268, the PBCRA locus was determined to be centromeric to D6S268. A recombination event in patient III:23 refined the telomeric region to marker D6S301 with data from III:24, IV:18, V:8 and V:9 determining the locus to be between D6S249 and D6S283. The marker that attained the highest maximum LOD score was D6S283, which is within the genomic region for *GRIK2*. Table 8.1 shows the two high-scoring markers used to link the disease to the 6q locus. The novel inter-photoreceptor matrix proteoglycan, *IMPG1* was mapped to 6q13-q15 and Gehrig et al. (1998) showed that no disease mutations in this gene were associated with the PBCRA family.

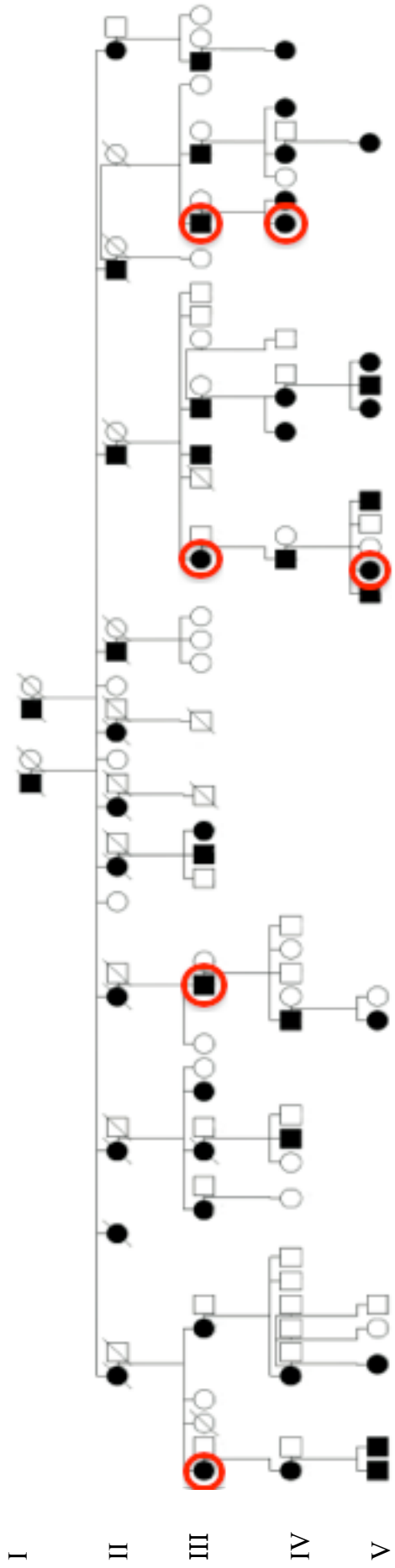


Figure 8.1 Pedigree of a five-generation family with progressive bifocal chorioretinal atrophy. Samples highlighted in red were analysed on a 250K Affymetrix *Sty* I chip.

Table 8.1 Information for the markers used to link the PBCRA disease locus.

Marker	Lod Score θ = 0	Physical Position	Annotations
D6S249	- ∞	98,117,908 – 98,118,062	Nearest gene C6orf167
D6S468	8.97	101,630,330 – 101,630,479	Between *ASCC3* and *GRIK2*
D6S283	10.02	102,359,767 – 102,360,017	Within *GRIK2*
D6S301	- ∞	103,694,956 – 103,695,197	

Prior to ending her term at the UCL Institute of Ophthalmology, Dr A. Kalhoro collaborated in the processing of DNA from six affected members of the original British family in Figure 8.1 (III:1, III:14, III:24, III:34, IV:24 and V:9) over the Affymetrix 250K *Sty* I GeneChips. The data analysis was not completed and so the analysis has been conducted as part of this project. The aim is to refine the 6q disease locus in the family in Figure 8.1 and in doing so identify candidate genes or possible disease-causing CNVs.

DNA from a small French family consisting of an affected parent and two affected offspring with a PBCRA-like phenotype will also be processed over the SNP chips and screened for mutations in candidate genes. Previous marker analysis data is not available for this family and the genotyping data will be compared to the PBCRA family to see if there are consistent disease alleles between the two families (indicating a common ancestral haplotype).

8.2 Methods Applied

8.2.1 Affymetrix 250K Protocol

The 250K Affymetrix protocol was followed as in Section 3.2.3, processing the samples circled in red in Figure 8.1

8.2.2 Candidate Gene Screening

MCHR2

Primers were designed by Dr A. Kalhoro and PCRs conducted using the BIOTaq protocol (Section 3.1.1). All amplicons were gel-extracted prior to sequencing. Primers and annealing temperatures are shown in Table 8.2.

Table 8.2 Primers and annealing temperatures for *MCHR2* coding exons.

Primers	Name	Annealing Temperature (°C)
ACCCTGGGATCCTGTGTTC	X2L	55
CCAAAATTGCATTTCATTAAGC	X2R	
CAAGACTAGGCATTTCCTCTAT	X3L	65
ACAAAGGAAGAAAACCTCTGC	X3R	
TTGCCCTGATAGAGGTGTGC	X4L	55
GGAGATACTGGCGAGAAGAGG	X4R	
TCTTGAGCTTTTCCACAAGG	X5L	55
ACATCTGTGATTTCAACACTCG	X5R	
TCCAGCTTTGGAAACTTTGG	X6AL	55
GTTTCCCATATTGTTGATTTCC	X6AR	
GGTGCTGGTGGTAGTCTTTATCC	X6BL	55
TGCATGGTTACACTTCTCTTCC	X6BR	
GCCTCAAATCCAAAGAAGAGC	X6CL	65
AGGGCAAGGTCAGGTTCC	X6CR	

FBXL4

Gradient BIOTaq PCRs were conducted for all primers (see Section 3.1.1) and PCRs then carried out at the optimal annealing temperature for each primer set using the BIOTaq protocol. Primers and annealing temperatures are shown in Section 5.3.2, Table 5.2.

SIM1

Gradient BIOTaq PCRs were conducted for all primers sets (Section 3.1.1) and PCRs then carried out at the optimal

annealing temperature for each primer set. The BIOTaq protocol was used and amplification products used directly in sequencing reactions unless otherwise stated (primers and annealing temperatures listed in Section 5.3.2, Table 5.3).

8.2.3 QPCR

QPCR experiments using SYBR Green were conducted to investigate copy number variations in affected individuals. The SYBR Green protocol was followed (Section 3.1.7). Primers for *GAPDH* (Table 8.3) were used in control reactions. The primers were tested for specificity in BIOTaq reactions using both the BIOTaq PCR cycles and the SYBR Green PCR cycles and a single amplicon was produced from each (Figure 8.2), which were sequenced for confirmation of the product.

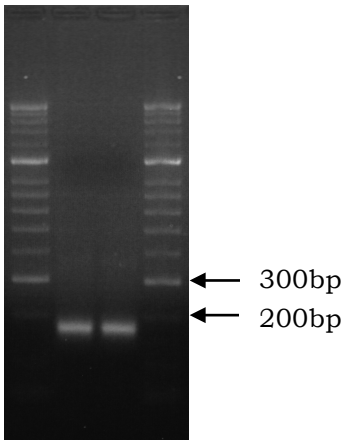


Figure 8.2 Gel image showing a single amplicon produced from BIOTaq and SYBR Green cycling schedules with *GAPDH* primers.

The test primers were used in BIOTaq gradient PCRs to identify the optimal annealing temperature and the amplicon was sequenced for confirmation of the product (Figure 8.3). The bands seen at the bottom of the gel represent the primers.

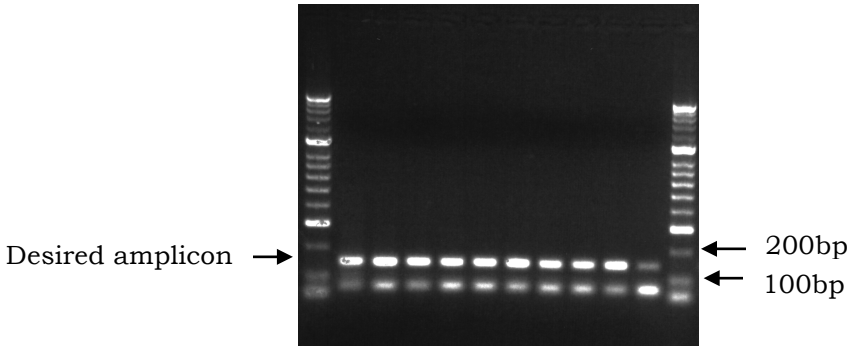


Figure 8.3 Gel image of the gradient BIOTaq PCRs conducted with the test primer set.

The PBCRA samples were diluted to a concentration of ~10ng/µl but due to the poor quality of the DNA accurate concentrations could not always be achieved. The primers are listed in Table 8.3.

Table 8.3 Primers used for PBCRA QPCRs.

Primers	Name
CATGCCATCACTGCCACCCAGAAGAC	GAPDH F
AGGCCATGCCAGTGAGCTTCCCGTTC	GAPDH R
CACATTCCAGAGCACATTGTTAATATCAC	Chr 6 CNV 1F
ATGGTGCCAATTGAGTGATTGTCACAGAA	Chr 6 CNV 1R

8.3 Results

8.3.1 Affymetrix Data

Genotyping Analysis

At 0.1 confidence the QC for each sample was >82%, whereas at 0.5 confidence it was >95%. Given the number of NoCalls at 0.1 confidence, the genotypes were extracted at 0.5 confidence.

As for MCDR1, MCDR3 and MCDR4, this analysis used only affected members but whereas MCDR3 and MCDR4 contained only affected parent-offspring samples, here affected parent-offspring samples have been used to determine the disease alleles in two cases. Two further samples have been run to increase the

meiotic events between samples and therefore more excluded SNPs are expected and alleles that are shared by all the members are likely to be more significant.

The PBCRA region was originally linked between D6S249 and D6S301 (Table 8.1). When the British family PBCRA AD for chromosome 6 was produced, many excludes were generated across the chromosome, which appeared to confirm and refine the disease region at 6q. Excludes across the entire chromosome were consistent in pattern and highlighted a long run of SNPs without excludes over 233 SNPs between rs1481454 (99,098,503) and rs9499580 (104,049,086), Figure 8.4.

III:34	IV:24	III:1	III:24	III:14	V:9	PBCRAAD	dbSNP RS ID
BB	BB	BB	BB	BB	BB	BB	rs17762161
AA	AA	AB	AB	AA	BB	Exclude	rs1904995
AB	AB	BB	AB	AA	BB	Exclude	rs11754417
AB	AB	AA	AA	AA	AA	AA	rs6929790
AA	AA	AA	AA	AA	AA	AA	rs958888
BB	BB	BB	BB	BB	BB	BB	rs1481459
BB	BB	BB	BB	BB	AB	BB	rs17769046
AB	AB	AB	AB	AA	BB	Exclude	rs1481453
AB	AB	AB	AB	BB	AA	Exclude	rs1481454
AA	AA	AA	AA	AA	AA	AA	rs9492007

III:34	IV:24	III:1	III:24	III:14	V:9	PBCRAAD	dbSNP RS ID
AA	AB	AB	AB	AA	AA	AA	rs7759394
AB	BB	AA	AA	AB	AB	Exclude	rs9499580
BB	BB	AB	BB	BB	BB	BB	rs9377535
AB	BB	AB	AA	AB	AB	Exclude	rs7452930
AB	AA	AB	BB	AB	AB	Exclude	rs9391078
AB	BB	AB	AA	AB	AB	Exclude	rs7744535
AB	BB	AB	AA	AB	AB	Exclude	rs9499614
AB	AA	AB	BB	AB	AB	Exclude	rs9499615
AB	AA	AB	BB	AB	AB	Exclude	rs9377543
AA	AA	AA	AB	AA	AA	AA	rs2501182
BB	BB	BB	BB	BB	BB	BB	rs2506732
AB	AA	AB	BB	AB	AB	Exclude	rs9373718

Figure 8.4 Excel sheet extracts showing the excludes that appear to define the disease region. The top image shows the excludes at the start of the region (the first SNP of the disease locus highlighted by the arrow) and the bottom image the excludes that appear to define the end of the disease region (identified with the arrow).

The majority of SNPs between these excludes are shown in Figures 8.5 to 8.7. In order to fit the data in more easily, some SNPs (those with homozygous calls for all samples) were removed but can be

seen in Appendix VI. Within the region between these SNPs was a single exclude output (Figure 8.6). Data extraction at 0.1 confidence provided three of the six samples with a NoCall at this SNP and the 0.5 exclude output was generated based on the genotypes of one these samples. As the Genotyping Console was unable to determine a genotype at 0.1 confidence at this SNP for half the samples and, more importantly, the sample for which the exclude was reliant on, the reliability of the exclude must be questioned. As this SNP lies in the middle of the region of consecutive SNPs that appear to segregate with the linked region (and therefore disease), it seems more likely that the exclude is not valid and the result of a miscall.

The disease region determined by the PBCRA AD genotypes suggests the locus begins at a similar point to the MCDR1 locus but extends beyond it. The markers D6S468 and D6S283, which achieved the highest significant LOD scores in the study linking PBCRA to chromosome 6q, lie within the disease region defined by the PBCRA AD (Figure 8.8).

Genotype Comparisons Between the Two Families

With a second suspected PBCRA family identified in France, the affected parent and offspring were analysed and the disease genotype for this family determined. This was then compared to the British PBCRA family to see if there may be a common ancestral mutation between the two families. If this were the case it would be expected that the two families would share alleles across the disease locus. At the newly refined PBCRA locus, regions of contiguous SNPs where alleles were shared between the two families were seen. These are highlighted in red boxes in Figures 8.5 – 8.8.

III:34	IV:24	III:1	III:24	III:14	V:9	PBCRA AD	V AD	dbSNP RS ID		
AA	AA	AA	AA	AA	AA	AA	AA	rs9492007		
AB	AB	BB	AB	BB	BB	BB	AA	rs12204275		
AB	BB	BB	BB	BB	AB	BB	AA	rs4839985		
AA	AA	AA	AA	AA	AB	AA	AA	rs1809181		POU3F2
AA	AA	AA	AA	AA	AA	AA	AA	rs2016597		
AA	AA	AA	AA	AA	AA	AA	AA	rs9375729		FBXL4
AA	AB	AA	AA	AB	AA	AA	AA	rs9385565		
AA	AB	AA	AA	AB	AA	AA	AB	rs9402366		
BB	BB	BB	AB	BB	AB	BB	AB	rs2180047		
BB	AB	BB	AB	AB	AB	BB	AB	rs2092769		
AB	AB	AB	AB	AB	AB	AB	AB	rs910423		
BB	AB	BB	AB	AB	AB	BB	AB	rs17614946		
BB	AB	BB	BB	AB	BB	BB	AA	rs1997937		
AA	AB	AA	AB	AB	AB	AA	BB	rs1884184		
AA	AB	AA	AB	AA	AB	AA	BB	rs9375909		
BB	AB	BB	AB	AB	AB	BB	AA	rs726488		
BB	BB	BB	BB	BB	BB	BB	AB	rs17059161		
AA	AA	AA	AA	AA	AA	AA	AB	rs17059192		
AB	AB	AB	AB	AB	AA	AA	BB	rs11154726		
AB	BB	AB	AB	BB	BB	BB	AA	rs4506054		
BB	BB	BB	BB	BB	BB	BB	BB	rs2173105		RP11-758C21.1
AB	BB	AB	AB	BB	BB	BB	AA	rs6569893		
BB	BB	BB	BB	BB	BB	BB	BB	rs1496972		
AB	BB	AB	AB	BB	BB	BB	AA	rs7767885		
AA	AA	AB	AA	AB	AA	AA	AA	rs12193294		D6S1717
BB	AB	AB	AB	AB	AB	BB	BB	rs6569950		
AA	AB	AA	AB	AA	AA	AA	AA	rs6909059		C6orf168
AA	AA	AA	AB	AA	AA	AA	AA	rs1988009		
AA	AB	AB	AB	AB	AB	AA	AA	rs7751616		
BB	BB	BB	AB	BB	BB	BB	BB	rs605277		
AB	AA	AA	AB	AA	AA	AA	AA	rs6924993		
BB	BB	BB	BB	BB	BB	BB	BB	rs4524617		
BB	BB	BB	BB	BB	BB	BB	BB	rs4144164		COQ3
BB	BB	AB	BB	BB	AB	BB	BB	rs12178596		
AA	AA	AA	AA	AA	AA	AA	AA	rs11965471		SFRS18
AB	BB	AB	BB	BB	AB	BB	AA	rs7776325		
AB	BB	BB	BB	BB	BB	BB	AA	rs13198933		RP11-9819.4
AB	BB	AB	BB	BB	AB	BB	AA	rs12198321		
AB	AB	AB	AB	AB	AB	AB	AA	rs4504482		USP45
AA	AA	AA	AA	AA	AA	AA	AA	rs10155760		
BB	BB	BB	BB	BB	BB	BB	BB	rs17059618		RP1-199J3.3
AB	AB	AA	AB	AB	AA	AA	AA	rs9402863		Y_RNA
AA	AA	AA	AB	AA	AA	AA	AA	rs485924		CCNC
AA	AA	AA	AB	AA	AA	AA	AA	rs182791		
BB	BB	BB	BB	BB	BB	BB	BB	rs17059663		PRDM13
BB	BB	BB	BB	BB	AB	BB	BB	rs17226753		
AA	AA	AA	AB	AA	AA	AA	AA	rs625011		Y_RNA
AB	AB	AB	AB	AB	AB	AB	AA	rs546567		
AB	AB	AA	AB	AB	AB	AA	AA	rs505908		
BB	BB	BB	BB	BB	AB	BB	BB	rs9389578		

SNPs that lie within gene regions

SNPs closest to markers

SNPs at which both families share alleles

Figure 8.5 Excel extract showing the genotypes for the British PBCRA samples compared to the French family (V) AD at the top of the disease region. Red boxes highlight runs of SNPs at which PBCRA and V AD share alleles.

III:34	IV:24	III:1	III:24	III:14	V:9	PBCRA AD	V AD	dbSNP RS ID		
BB	BB	BB	BB	BB	AB	BB	BB	rs9389578		
AB	BB	AB	BB	BB	BB	BB	BB	rs10485223		
AB	BB	BB	AB	BB	AB	BB	AA	rs4527714		
BB	BB	BB	BB	BB	AB	BB	AA	rs17059831		
AB	AB	BB	AB	AB	AB	BB	AA	rs7451834		
AA	AB	AA	AA	AA	AB	AA	BB	rs9495587		
AB	AB	AB	AB	AB	AB	AB	BB	rs9495655		
AB	AB	AB	AB	AB	AB	AB	BB	rs4565312		
AB	AB	AB	AB	AB	AB	AB	AA	rs9495667		
AB	AA	AA	AA	AB	AA	AA	BB	rs9389810		
AB	AB	BB	BB	AB	BB	BB	BB	rs4320381		
AB	AB	AB	AB	AB	AB	AB	AA	rs2397670		
AB	BB	AB	AB	AB	AB	BB	AA	rs10499028		
BB	BB	AB	BB	BB	AB	BB	AA	rs9376547		MCHR2
BB	BB	BB	BB	BB	BB	BB	BB	rs2397693		
BB	BB	AB	BB	BB	BB	BB	BB	rs6919506		RP11-1414.3
AA	AA	AB	AA	AA	AA	AA	AA	rs4240586		RP11-1414.2
AA	AA	BB	AA	AA	AA	Exclude	AA	rs17789218		RP3-344J20.2
AA	AB	AA	AB	AA	AA	AA	AB	rs2474267		
AA	AA	AB	AA	AA	AA	AA	BB	rs12201659		RP3-344J20.1
AA	AB	AB	AA	AA	AB	AA	BB	rs714606		
AB	AB	AB	AB	BB	AB	BB	AA	rs8180548		
AA	AB	AB	AB	AA	AB	AA	BB	rs9321986		
BB	AB	AB	BB	BB	AB	BB	AA	rs2841308		
BB	AB	AB	AB	AB	AB	BB	AA	rs17792460		
BB	BB	BB	BB	AB	BB	BB	AB	rs17793103		
AB	AB	AB	AA	AB	AB	AA	BB	rs17060460		SIM1
AA	AB	AA	AA	AA	AB	AA	AA	rs3798492		RP1-121G13.2
BB	AB	BB	BB	AB	BB	BB	BB	rs2157342		
BB	AB	BB	BB	AB	BB	BB	BB	rs6903206		ASCC3
AA	AB	AA	AA	AA	AA	AA	AA	rs2398132		RP1-121G13.3
BB	AB	BB	BB	BB	BB	BB	BB	rs7452753		
AA	AB	AB	AA	AA	AA	AA	AA	rs11155596		ASCC3
AA	AA	AA	AA	AA	AA	AA	AA	rs2055162		
AB	AB	AB	AB	AB	AB	AB	BB	rs617365		
AB	AB	AB	AB	AB	AB	AB	AA	rs596601		snoU13
AB	BB	AB	AB	AB	AB	BB	BB	rs1406894		
AB	AB	AB	AB	AB	AB	AB	AA	rs4432986		
AB	AB	AB	AB	AB	AB	AB	AA	rs9404084		
AA	AA	AA	AA	AA	AA	AA	AA	rs7757359		
AB	AB	AB	AB	AB	AB	AB	BB	rs2813643		
AB	AB	AB	AB	AB	AB	AB	BB	rs2209936		
AB	AB	AB	AB	AB	AB	AB	BB	rs2149461		
AA	AA	AA	AA	AA	AA	AA	AA	rs17061486		D6S468
AB	AB	AB	AB	AB	BB	BB	AB	rs406541		
BB	BB	BB	BB	BB	BB	BB	BB	rs166885		
AB	AB	AB	BB	AB	AB	BB	AB	rs17061665		
AB	AB	AB	AB	AB	BB	BB	AB	rs1485833		
AB	AB	AB	AB	AB	AB	AB	AB	rs17054559		
AB	AB	AB	AB	AB	BB	BB	AB	rs1485840		
AA	AA	AA	AA	AA	AA	AA	AA	rs1485841		GRIK2

SNPs that lie within gene regions

SNPs closest to markers

SNPs at which both families share alleles

Figure 8.6 Excel image showing the British PBCRA sample genotypes and generated disease AD compared to the French family (V) AD over the middle of the disease region. Red boxes highlight shared alleles between PBCRA and V AD genotypes.

III:34	IV:24	III:1	III:24	III:14	V:9	PBCRA AD	V AD	dbSNP RS ID		
BB	BB	BB	BB	BB	BB	BB	BB	rs166885		
AB	AB	AB	BB	AB	AB	BB	AB	rs17061665		
AB	AB	AB	AB	AB	BB	BB	AB	rs1485833		
AB	AB	AB	AB	AB	AB	AB	AB	rs17054559		
AB	AB	AB	AB	AB	BB	BB	AB	rs1485840		
AA	AA	AA	AA	AA	AA	AA	AA	rs1485841		GRIK2
AB	AB	AB	BB	AB	AB	BB	AB	rs2245037		RP11-93K7.1
AB	AB	AB	AB	AB	AB	AB	BB	rs17760780		
AA	AA	AA	AA	AA	AA	AA	AA	rs2852584		GRIK2
AA	AA	AA	AA	AA	AA	AA	AA	rs12193068		
AB	BB	BB	BB	BB	BB	BB	AB	rs1415483		
AB	AA	AB	AA	AA	AA	AA	AB	rs2105118		
AA	AA	AA	AA	AA	AA	AA	BB	rs942666		
BB	BB	BB	BB	BB	BB	BB	AB	rs17054632		
BB	BB	AB	BB	BB	BB	BB	AA	rs17062449		
BB	BB	BB	BB	BB	AB	BB	AB	rs2518203		
BB	BB	BB	BB	BB	BB	BB	BB	rs1340274		D6S283
BB	BB	BB	BB	BB	BB	BB	BB	rs9322622		
AA	AA	AA	AA	AA	AA	AA	AA	rs6923395		
BB	BB	BB	BB	BB	BB	BB	BB	rs6928180		
AB	BB	AB	BB	BB	AB	BB	BB	rs7749722		
AB	AA	AA	AA	AA	AB	AA	AA	rs2782920		AP002530.2
AA	AB	AA	AB	AA	AA	AA	AA	rs9485620		
AB	AB	AA	AB	AB	AB	AA	BB	rs6902587		
AA	AB	AB	AB	AB	AB	AA	BB	rs1232230		
BB	AB	AB	AB	AB	AB	BB	AA	rs9498995		
BB	AB	AB	AB	AB	AB	BB	AA	rs9390817		
AB	AB	AB	AB	AB	AB	AB	AA	rs7759092		
AB	BB	AB	BB	AB	AB	BB	AA	rs9377361		
AA	AA	AA	AA	AB	AA	AA	BB	rs9377366		RP11-793L10.1
AB	AB	AA	AB	AA	AA	AA	AA	rs680011		
BB	BB	AB	BB	BB	AB	BB	BB	rs9499177		
BB	BB	AB	BB	BB	AB	BB	BB	rs9499178		
AB	AB	AB	AB	AA	AB	AA	BB	rs1996911		
AB	AB	AB	BB	AB	AB	BB	BB	rs6918215		
AB	AB	AB	AA	AB	AB	AA	AA	rs9404274		
AB	AB	AB	AA	AB	AB	AA	AA	rs9377412		
AB	AB	BB	AB	BB	AB	BB	AB	rs9373672		
AB	AA	AA	AA	AA	AA	AA	AA	rs12192767		
AB	AB	AA	AA	AA	AA	AA	AA	rs6571083		
BB	BB	AB	AB	AB	BB	BB	BB	rs9373682		
AB	AA	AB	AB	AA	AA	AA	AA	rs6571094		D6S301
AB	AA	AA	AA	AA	AA	AA	AA	rs2143795		
AB	AA	AA	AB	AA	AA	AA	AB	rs9404372		
AB	AA	AA	AB	AA	AA	AA	AB	rs9499431		
AB	AA	AA	AB	AA	AA	AA	AB	rs2207021		
AB	AA	AB	AB	AB	AB	AA	BB	rs2503296		
AB	AB	BB	AB	AB	AB	BB	BB	rs2506743		
AA	AB	AB	AB	AA	AA	AA	AA	rs4947151		
BB	AB	AB	AB	BB	BB	BB	BB	rs9391057		
AA	AB	AB	AB	AA	AA	AA	AA	rs7759394		SNORA33

SNPs that lie within gene regions

SNPs closest to markers

SNPs at which both families share alleles

Figure 8.7 Excel extract showing the British PBCRA sample genotypes and generated disease AD compared the French family (V) AD at the end of the PBCRA disease region. Red boxes highlight shared alleles

The dominant genotype (AD) for the French family was compared to the British and the genotypes of the two families were 85% identical at the new PBCRA locus, with just 36 of the 233 AD alleles in the suspected disease region differing between the British (labelled PBCRA) AD and French (labelled V) AD. Throughout the rest of chromosome 6 many exclude outputs were given, indicating differences between the British and French genotypes. Of a total 14,129 SNPs on chromosome 6, 5,291 provided exclude outputs, meaning that the overall identity of the families chromosome 6 genotypes were 63% compared to 85% at the disease locus.

The largest region of contiguous shared alleles between the two families occurred between rs3798492 (100,888,625) and rs2105118 (102,113,988), shown in Figure 8.8. This indicates there could be a shared haplotype between these two families if this turns out to be the precise location of the genetic error causing PBCRA.

To provide further evidence of linkage of the French family to this region, a search for low frequency alleles that segregate with the disease was made. Given that in the general population the frequency of one allele is much lower than the other, for it to be seen in both families may suggest segregation of the allele with the disease. There were many SNPs at which a number of the samples had heterozygous calls but for the majority the frequency of alleles was around 50:50. Five rare alleles were identified in both families, which are in the latter half of the disease region and listed in Table 8.4,

III:34	IV:24	III:1	III:24	III:14	V:9	PBCRA AD	V AD	dbSNP RS ID		
AA	AB	AA	AA	AA	AB	AA	AA	rs3798492		RP1-121G13.2
BB	AB	BB	BB	AB	BB	BB	BB	rs2157342		
BB	AB	BB	BB	AB	BB	BB	BB	rs6903206		ASCC3
AA	AB	AA	AA	AA	AA	AA	AA	rs2398132		RP1-121G13.3
BB	AB	BB	BB	BB	BB	BB	BB	rs7452753		
AA	AB	AB	AA	AA	AA	AA	AA	rs11155596		ASCC3
AA	AA	AA	AA	AA	AA	AA	AA	rs2055162		
AB	AB	AB	AB	AB	AB	AB	BB	rs617365		
AB	AB	AB	AB	AB	AB	AB	AA	rs596601		snoU13
AB	BB	AB	AB	AB	AB	BB	BB	rs1406894		
AB	AB	AB	AB	AB	AB	AB	AA	rs4432986		
AB	AB	AB	AB	AB	AB	AB	AA	rs9404084		
AA	AA	AA	AA	AA	AA	AA	AA	rs7757359		
AB	AB	AB	AB	AB	AB	AB	BB	rs2813643		
AB	AB	AB	AB	AB	AB	AB	BB	rs2209936		
AB	AB	AB	AB	AB	AB	AB	BB	rs2149461		
AA	AA	AA	AA	AA	AA	AA	AA	rs17061486		D6S468
AB	AB	AB	AB	AB	BB	BB	AB	rs406541		
BB	BB	BB	BB	BB	BB	BB	BB	rs166885		
AB	AB	AB	BB	AB	AB	BB	AB	rs17061665		
AB	AB	AB	AB	AB	BB	BB	AB	rs1485833		
AB	AB	AB	AB	AB	AB	AB	AB	rs17054559		
AB	AB	AB	AB	AB	BB	BB	AB	rs1485840		
AA	AA	AA	AA	AA	AA	AA	AA	rs1485841		GRIK2
AB	AB	AB	BB	AB	AB	BB	AB	rs2245037		RP11-93K7.1
AB	AB	AB	AB	AB	AB	AB	BB	rs17760780		
AA	AA	AA	AA	AA	AA	AA	AA	rs2852584		GRIK2
AA	AA	AA	AA	AA	AA	AA	AA	rs12193068		
AB	BB	BB	BB	BB	BB	BB	AB	rs1415483		
AB	AA	AB	AA	AA	AA	AA	AB	rs2105118		
AA	AA	AA	AA	AA	AA	AA	BB	rs942666		
BB	BB	BB	BB	BB	BB	BB	AB	rs17054632		
BB	BB	AB	BB	BB	BB	BB	AA	rs17062449		
BB	BB	BB	BB	BB	AB	BB	AB	rs2518203		
BB	BB	BB	BB	BB	BB	BB	BB	rs1340274		D6S283
BB	BB	BB	BB	BB	BB	BB	BB	rs9322622		
AA	AA	AA	AA	AA	AA	AA	AA	rs6923395		
BB	BB	BB	BB	BB	BB	BB	BB	rs6928180		
AB	BB	AB	BB	BB	AB	BB	BB	rs7749722		
AB	AA	AA	AA	AA	AB	AA	AA	rs2782920		AP002530.2
AA	AB	AA	AB	AA	AA	AA	AA	rs9485620		

SNPs that lie within gene regions

SNPs closest to markers

SNPs at which both families share alleles

Figure 8.8 The possible PBCRA disease region determined from 250K genotype comparisons between a British and French family. Shared alleles are highlighted in the red boxes.

Table 8.4 SNPs of low frequency alleles that segregate with the disease.

SNP ID	Physical Position	Allele Frequencies	PBCRA & VAD Allele	Nearby Gene/ Marker
rs505908	100,111,078	A = 0.325 G = 0.675	A	
rs617365	101,351,365	T = 0.817 C = 0.183	T/C	D6S468
rs406541	101,714,615	A = 0.783 C = 0.217	C	D6S468
rs17054559	101,813,148	A = 0.033 G = 0.967	A/G	*GRIK2*
rs9373672	103,463,745	T = 0.675 G = 0.325	G	

It appears that SNPs between rs1481454 and rs9499580 segregate with the disease in the British PBCRA family. The French family with a PBCRA-like phenotype shows segments within this PBCRA locus where contiguous disease alleles match those of the British PBCRA family. Low frequency alleles segregate with the disease locus in both families. These combined data are consistent with a common origin of mutation in the two families. Previous marker scores and low frequency allele segregation suggest the latter half of the disease region could be more important in the search for candidate genes. Further evidence for linkage of the French family to this region and a single haplotype existing between both families is shown in Figure 8.8. Here, the longest run of contiguous shared alleles between affecteds of both families is shown and is suggestive of a common haplotype and therefore mutation event within the region. Candidate genes in this region include *SIM1, ASCC3* and *GRIK2*. Of these, only *GRIK2* expression in the RPE/choroid is reported on the NEIBank database. *ASCC3* is not revealed in the database to have expression in either the retina or RPE/choroid, but *ASCC3-like 1* is, which suggests a role for *ASCC3* is not unlikely. Expression of *SIM1* is not reported in either database.

Copy Number Analysis

The CNV data were determined to be in bounds for the French family samples but none of the CNVs identified were consistent amongst the family members. Data for all the British PBCRA samples were out of bounds but analysis was conducted despite this to give an indication of possible CNV regions that could be investigated further.

Numerous common CNVs were identified across the genome, many of which overlap with known regions of CNV. These are shown in Table 8.5 with those that overlap with genes or known CNVs indicated. The genes within regions of CNVs common to all family members were investigated for expression in the retina or RPE/choroids on NEIBank or general eye expression (Unigene). Of the genes listed in these databases just six have published roles in the eye described. Of these, just one does not occur in a region where CNVs have previously been described in "normal" individuals. The only CNV unique to the British PBCRA family is on chromosome 14q23.1 and results in a deletion of one copy of *ARID4A* (AT-rich interactive domain 4A (RBP1-like)). This CNV was not seen in any of the French family members. Although there are regions of CNV seen on chromosome 6 in members of this family, none are at the linked disease locus. A CNV at *EYS* is interesting because mutations in this cause RP (Abd El-Aziz et al., 2008) but variations at this genomic region have been reported in the DGV and are therefore unlikely to be significant.

Table 8.5 CNVs identified in all members of the British PBCRA family analysed.

Chromosome	CN State	First shared SNP	Last shared SNP	CNV in dGV	Genes within CNV	Unigene Eye Expression	EyeSAGE Retina	EyeSAGE RPE/Choroid	Published Roles
1	1	rs1931328	rs16850436						
	3	rs3002680	rs11264123		*SFPQ*, splicing factor proline/glutamine-rich	*SFPQ*			
		rs4433364	rs17129533		*INSL5*, insulin-like 5, *WDR78*, WD repeat domain 78, *MIER1*, mesoderm induction early response 1 homolog	*WDR78, MIER1*	*MIER1*	*WDR78*	
2	1	rs17045365	rs10179985						
	3	rs16846047	rs2892792		*SLC4A10*, solute carrier family 4, sodium bicarbonate transporter, member 10,	*SLC4A10*			
		rs2628473	rs11127111		*ANKRD36BP2*, ankyrin repeat domain 36B pseudogene 2,				
		rs1348853	rs7355478		*EPC2*, enhancer of polycomb homolog 2	*EPC2*			
		rs10931160	rs2216837						
		rs4667231	rs11683183		*GULP*, engulfment adaptor PTB domain containing 1	*GULP*			
		rs16843614	rs11688922	Korbel et al., (2007), Kidd et al., (2008)	*MAP2*, microtubule-associated protein 2, *UNC80*, unc-80 homolog, *RPE*, ribulose-5-phosphate-3-epimerase	*MAP2*			Late neuronal marker, marker for RGC subset
3	1	rs2730363	rs17272285						
		rs13433980	rs6439655		*STAG1*, stromal antigen 1	*STAG1*			
	3	rs1384300	rs7426534						
		rs1112475	rs9874551		*EPHA6*, EPH receptor A6				
		rs6802644	rs1380786						
		rs9812602	rs526844						
4	1	rs17015895	rs3775443	Kidd et al., (2008), Itsara et al., (2009), Jakobsson et al., (2008)	*SNCA*, synuclein, alpha (non A4 component of amyloid precursor)	*SNCA*			Found in neurons and associated with neurodegenerative diseases
	3	rs12502062	rs4832858						
		rs11931045	rs16856781		Upstream of *KCTD8*, potassium channel tetramerisation domain containing 8	*KCTD8*			
		rs11097327	rs17019324		Start of *GRID2*, glutamate receptor, ionotropic, delta 2				
5	3	rs4866976	rs8188245	Cooper et al., (2008), Perry et al., (2008), Itsara et al., (2009), Korbel et al., (2007), Conrad et al., (2009)	*HCN1*, hyperpolarisation activated cyclic nucelotide-gated potassium channel 1	*HCN1*			Found in photoreceptors and bipolar cells
		rs246902	rs390694		C5orf30	*C5orf30*			
		rs962681	rs6875297		*FBXL17*, F-box and leucine-rich repeat protein 17	*FBXL17*			
		rs6874509	rs10053787		*ISOC1*, isochorismatase domain containing 1	*ISOC1*	*ISOC1*		
6	3	rs9363384	rs6941349	Perry et al., (2008), Jakobsson et al., (2008), Itsara et al., (2009), Redon et al., (2006), Kidd et al., (2008)	*EYS*, eyes shut homolog	*EYS*			Mutations cause AR RP, CORD, rod-cone and cone dystrophies
		rs9398678	rs6569287		*PKIB*, protein kinase (cAMP-dependent, catalytic) inhibitor beta, *FAB7*, fatty acid binding protein 7, brain, *SMPDL3A*, sphingomyelin phosphodiesterase, acid-like 3A	*PKIB, SMPDL3A*			
		rs9385452	rs17055886		*PTPRK*, protein tyrosine phosphatase, receptor type, K	*PTPRK*			
7	1	rs1534378	rs7799581						
		rs4144090	rs2536149		C7orf58	*C7orf58*			
	3	rs10277093	rs41447						
		rs1912445	rs17169304		*ASNS*, asparagine synthetase (glutamine-hydrolyzing), *OCM2*, oncomodulin 2	*ASNS*		*ASNS*	
8	3	rs4272377	rs1478041						
9	3	rs4745138	rs942396		C9orf85, *HSPB1P1*, heat shock 27kDa protein 1 pseudogene 1	*C9orf85*			
10	3	rs9420431	rs10466230		*GLUD1*, glutamate dehydrogenase 1, *FAM35A*, family with sequence similarity 35, member A	*GLUD1, FAM35A*		*FAM35A*	
12	1	rs11056433	rs2302687	Korbel et al., (2007), Conrad et al., (2009), Kidd et al., (2008)	*PTPRO*, protein tyrosine phosphatase, receptor type, O	*PTPRO*			Involved in neuronal guidance
	3	rs11246908	rs11246940		*EP400*, E1A binding protein p400, *EP400NL*, EP400 N-terminal like	*EP400, EP400NL*		*EP400*	
14	1	rs2348073	rs7151036		*ARID4A*, AT rich interactive domain 4A (RBP1-like)	*ARID4A*			Expressed in developing neuroretina
	3	rs7154462	rs10137879						
16	3	rs8062405	rs12596913	Redon et al., (2006), Itsara et al., (2009)	*ATXN2L*, ataxin 2-like, *TUFM*, Tu translation elongation factor, mitochondrial, *SH2B1*, SH2B adaptor protein 1, ATPase, Ca++ transporting, cardiac muscle, fast twitch 1	*ATXN2L, TUFM, SH2B*	*SH2B1*	*ATXN2L, SH2B1*	
		rs11075782	rs7204496	Itsara et al., (2009) (across *RBL2*, not *CDH9*)	*CHD9*, chromodomain helicase DNA binding protein 9, *RBL2*, retinoblastoma-like 2	*CHD9, RBL2*		*CHD9*	
		rs7187262	rs16958951	Kidd et al., (2008) (but not at *CYB5B*)	*CYB5B*, cytochrome b5 type B (outer mitochondrial membrane)	*CYB5B*		*CYB5B*	
17	1								
	3	rs1106175	rs7213702		*DOC2B*, double C2like domains, beta, *RPH3AL*, rabphilin 3A-like (without C2 domains)	*DOC2B, RPH3AL*			
20	3	rs6120998	rs2297849		*SPAG4*, sperm associated antigen 4, *CPNE1*, copine 1, *NFS1*, NFS1 nitrogen fixation 1 homolog (S. cerevisiae), *RBM12*, RNA binding motif protein 12	*SPAG4, CPNE1, NFS1*	*SPAG4, CPNE1*	*CPNE1*	
21	1	rs4817011	rs1475886						
	3	rs2825778	rs1971505						

At the start of the 6q disease region between SNPs rs9398847 (99,026,809) and rs12204275 (99,138,875), five of the six British PBCRA family samples were determined to have a copy number state of one (none of the French family members had a CNV in this region). With no samples being of the recommended quality for CNV analysis it was deemed worthwhile to further investigate this. Despite the CNV analysis detecting a CN state of 1 across many SNPs in five of the six samples, there was no evidence in the genotyping data of a loss of alleles in this region. If a deletion were present, then it would be expected that runs of homozygous SNPs would be evident at the deleted SNPs but there were in fact numerous heterozygous calls between rs3125579 and rs12204275 (Figure 8.9).

III:34	IV:24	III:1	III:24	III:14	V:9	PBCRAAD	dbSNP RS ID
BB	**BB**	**AB**	**BB**	AB	BB	BB	rs3125579
AA	**AA**	**AA**	**AA**	AB	AA	AA	rs9491645
AB	**BB**	**BB**	**BB**	BB	BB	BB	rs210405
BB	**BB**	**BB**	**BB**	**AB**	BB	BB	rs210397
AB	**BB**	**AB**	**BB**	**BB**	BB	BB	rs211214
AB	**BB**	**AB**	**BB**	**BB**	BB	BB	rs183317
AB	**AA**	**AB**	**AA**	**AA**	AA	AA	rs638458
BB	**BB**	**BB**	**BB**	**BB**	BB	BB	rs9398847
AA	**AA**	**AA**	**AA**	**AA**	AA	AA	rs9401968
BB	**BB**	**BB**	**BB**	**BB**	BB	BB	rs9388585
BB	**BB**	**BB**	**BB**	**BB**	BB	BB	rs17762161
AA	**AA**	**AB**	**AB**	**AA**	BB	Exclude	rs1904995
AB	**AB**	**BB**	**AB**	**AA**	BB	Exclude	rs11754417
AB	**AB**	**AA**	**AA**	**AA**	AA	AA	rs6929790
AA	**AA**	**AA**	**AA**	**AA**	AA	AA	rs958888
BB	**BB**	**BB**	**BB**	**BB**	BB	BB	rs1481459
BB	**BB**	**BB**	**BB**	**BB**	AB	BB	rs17769046
AB	**AB**	**AB**	**AB**	**AA**	BB	Exclude	rs1481453
AB	**AB**	**AB**	**AB**	**BB**	AA	Exclude	rs1481454
AA	**AA**	**AA**	**AA**	**AA**	AA	AA	rs9492007
AB	**AB**	**BB**	**AB**	**BB**	BB	BB	rs12204275

SNPs with AB call

Figure 8.9 Excel sheet extract of British PBCRA genotypes at the SNPs where the CNV analysis detected loss of one allele. Blue highlights AB calls in the suspected region of deletion. Bold letters indicate SNPs where a loss of allele was detected by CNV analysis.

Heterozygous calls in the genotyping data at the region of the apparent CNV suggest the deletion is not genuine. If one allele were deleted across a number of SNPs then homozygous calls would be expected across that region. However, the genotyping data were also not as reliable as desired as they were extracted at

0.5 confidence and with the inconsistencies between the two analyses, QPCRs were conducted to determine whether the PBCRA samples did have a region of deletion at 6q16.1.

8.3.2 QPCR

Given the contradiction between the CNV and genotyping analysis, QPCRs were conducted to investigate the apparent deletion present prior to the PBCRA disease region in five of the six samples, as determined by the Affymetrix CNV analysis. *GAPDH* control QPCRs were conducted on the same plate as the test QPCR, which amplified a 150bp fragment between SNPs rs9388585 (99,027,155) and rs17762161 (99,028,910), Figure 8.10.

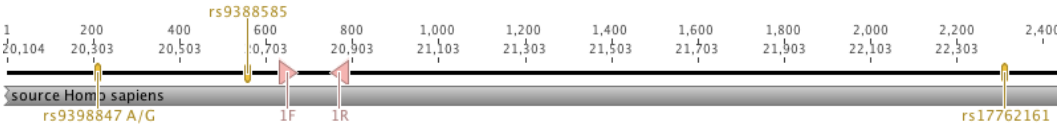


Figure 8.10 Graphic showing the relative position of the QPCR test primers in relation to three 250K SNPs.

The delta-delta Ct method was used to determine copy number states (Section 4.5) with the normalised CN state shown in Figure 8.11 (1 represents the normal CN state). Error bars show standard error of the mean (SEM) taken from an average of three reactions for all samples. ANOVA revealed no significant difference between the Control and test sample group ($p = 0.456$). Whilst sample III:14 appears to have less DNA than the other samples, t-test analysis revealed no significant difference compared to the Control ($p = 0.222$). The statistical analysis indicates all samples have the same CN state and therefore there is no CNV present in the PBCRA samples at this region.

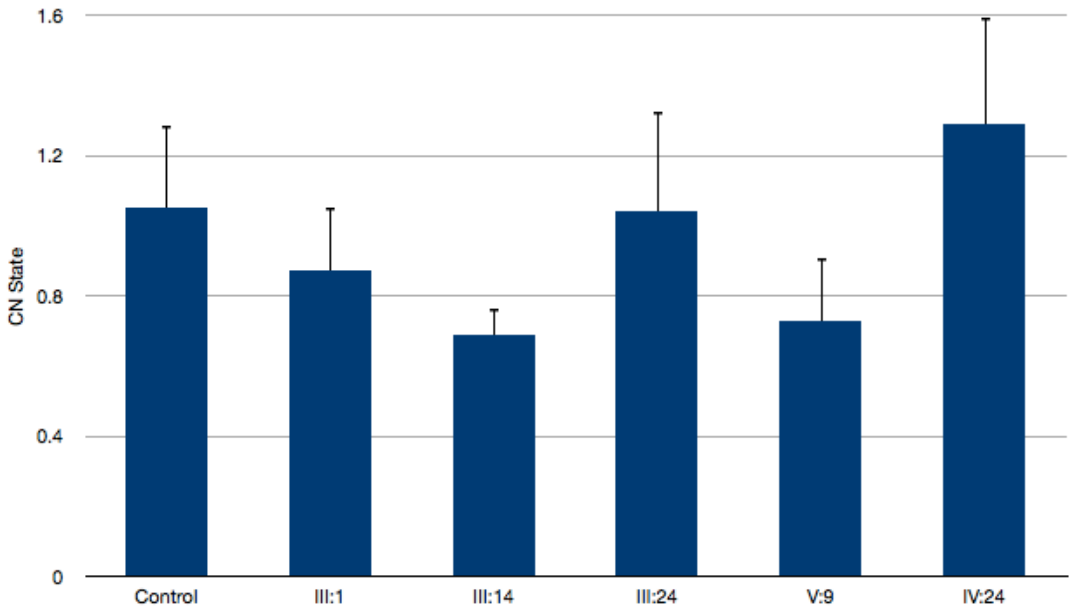


Figure 8.11 Graph showing the delta-delta-Ct values for the chromosome 6 test region. Error bars show SEM.

8.3.3 Candidate Screening

MCHR2

This gene appeared to be a good candidate as it is known not to be present in the equivalent region of the mouse genome and mice lack a macula, suggesting it may have a role within in the human macula. In the six PBCRA samples and three French samples screened, the coding exons (2 to 6) were analysed for mutations but none were found. The variations seen are shown in Table 8.6. Attempts to amplify the 5' UTR exon 1 failed despite using different primers sets and reaction conditions.

Table 8.6 SNPs identified in *MCHR2* in the British and French PBCRA samples.

Exon/Intron	SNP ID	Variation	Number of British PBCRA Affecteds with SNP	Number of French PBCRA Affecteds with SNP
3	rs74531963	A = 0.850 G = 0.150		3
6 (3' UTR)	rs4839764	A = 0.999 G = 0.001	3	3

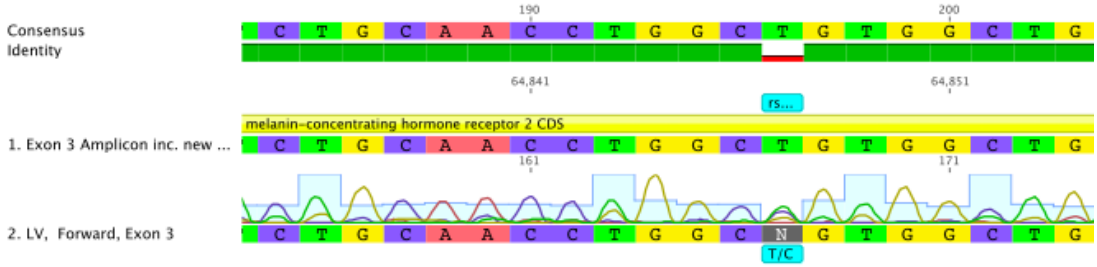


Figure 8.12 Sequencing example of *MCHR2* exon 3 from a French family member showing SNP rs74531963.

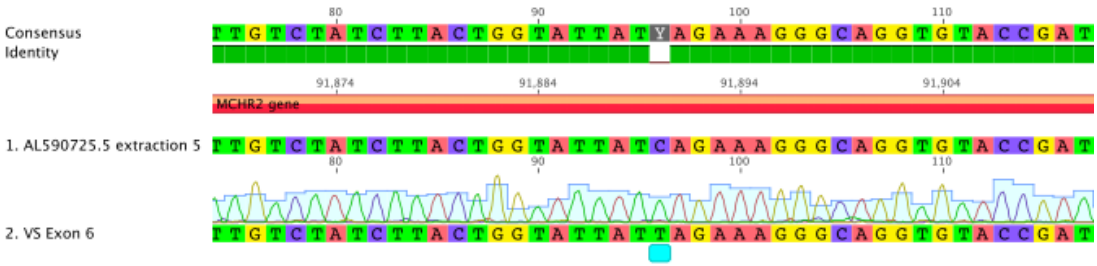


Figure 8.13 Sequencing data example showing the *MCHR2* SNP rs4839764 in a member of the British PBCRA family.

FBXL4* and *POU3F2

The genotyping comparisons between the British and French families identified a region of similarity in the genomic region for *FBXL4* and *POU3F2*. Despite identifying variations within the coding exons of *FBXL4*, none were unique to affected individuals. These are shown in Table 8.7. The promoter and exon 1 sequences failed to amplify, which was also the case for control and MCDR1 samples.

Attempts were made to screen *POU3F2* but failed despite numerous primer design attempts, using different reagents and reaction conditions.

Table 8.7 SNPs identified in *FBXL4* in the British and French PBCRA samples.

Exon/Intron	SNP ID	Variation	Number of British PBCRA Affecteds with SNP	Number of French PBCRA Affecteds with SNP
Exon 2 (5' UTR)	rs195812	A = 0.608 C = 0.392	1	2, also seen in control and MCDR1 samples
Exon 3	rs1011676	G = 0.929 A = 0.071		1
Intron 5	rs6935315	A = 0.572 G = 0.428	1	3, also seen in MCDR1 samples
Exon 8		T/A	1	

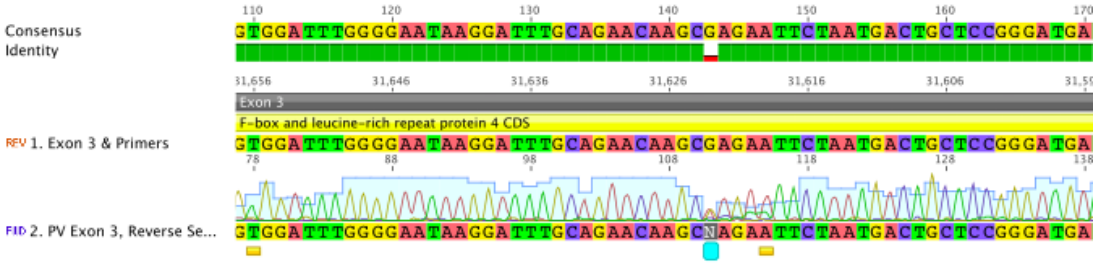


Figure 8.14 Sequencing data for a French family member carrying a variation at rs1011676 in exon 3 of *FBXL4*.

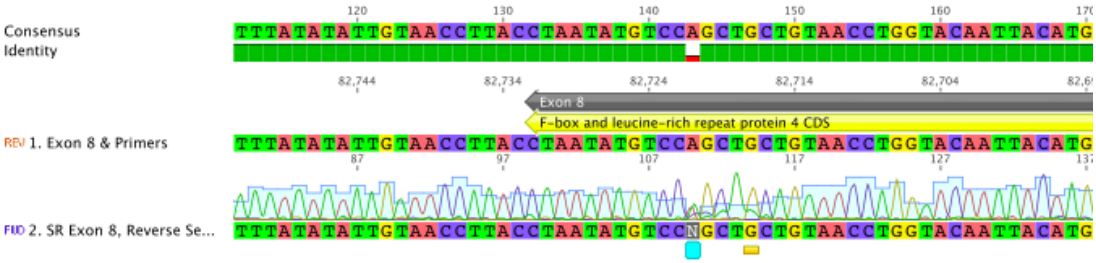


Figure 8.15 Sequencing data from exon 8 of *FBXL4* of a British family member showing a SNP.

SIM1

This was identified as a possible candidate in the PBCRA genotyping analysis comparisons with the French family AD. Mutation screening in all coding exons and the promoter region revealed no variations in the PBCRA or French family samples screened.

8.4 Discussion

8.4.1 Genotyping Analysis

The Affymetrix analysis for PBCRA has focused on affected individuals only, five individuals from the original British family and three individuals from the French family. By analysing parent-offspring pairs this has enabled not only determination of a PBCRA haplotype at the disease region but also extensive data on CNVs across a number of affected individuals. In the large British family, the affecteds have been selected from across the pedigree thereby comparing distant relatives, which increases the chance of refining the disease region. Previously linked to 6q14-q16.3, the genotype comparisons here used excluded SNPs (those at which the affected related members do not share the same allele on their disease chromosome) to refine the region to 6q16.1-q16.3. This is a direct overlap with the MCDR1 locus. The British PBCRA AD genotypes were compared to the disease AD genotypes generated from a small French family with a phenotype consistent with PBCRA. Linkage in this French family has not previously been conducted due to its small size (affected parent and two affected offspring). Comparisons of the chromosome 6 AD genotypes did not exclude linkage of the French family to the 6q16.1-q16.3 region. The AD genotypes showed the two families shared 85% AD alleles at the disease region (compared to 63% across the whole chromosome), including low frequency alleles

that appear to segregate with the disease (Table 8.4). The region of high similarity at the 6q16.1-q16.3 between the British and French AD alleles, which is not seen across the remainder of chromosome 6, suggests alleles in this region may be segregating with the disease in both families, which implies there is a common origin of disease. Further evidence of this came from the genotyping comparisons revealing the longest run of contiguous alleles shared between the two families was between rs3798492 and rs2105118. This region of shared alleles suggests the disease region could be refined to this part of 6q16.2, which contains *SIM1, ASCC3,* the pseudogene RP1-121G13.3, snoU13, *GRIK2* and the pseudogene RP11-93K7.1. In addition to this evidence, the highest scoring marker from the previous linkage analysis conducted by Kelsell et al. (1998) was within the genomic region for *GRIK2* and this is the only gene for whom expression is shown in the RPE/choroids database (NEIBank).

Candidates other than *GRIK2* have been considered in Section 5.5.3 and will not be repeated here as the same points are valid for this disorder. Though *MCHR2* was not highlighted by the comparison of the AD genotypes from the two families, screening was conducted because it was highlighted previously as an interesting candidate and screened in MCDR1 samples. The British PBCRA family showed no mutations in the coding exons of this gene. The French family samples revealed a heterozygous change in exon 3 but this is not considered to be important to the disease state as it represents a synonymous coding change. *SIM1* was also screened in the two families but identified no mutations or variations.

GRIK2 encodes glutamate receptor, ionotropic, kainate 2, which is an important neurotransmitter receptor in the central nervous

system. Mutations in this gene have been shown to cause autosomal recessive mental retardation (Motazacker et al., 2007) and are associated with age of onset of Huntington disease (Rubinsztein et al., 1997). *GRIK2* was identified in amacrine cells of the adult retina by Brandstätter et al. (1994) and Vardi et al. (1998) showed GRIK2 receptors concentrate on dendrites of bipolar cells in monkey and rat retina. Grünert et al. (2003) showed GRIK2 is predominantly associated with diffuse bipolar cells and rod bipolar cells in primate retina. Based on these findings, GRIK2 is likely to be important in the retina despite other mutations in *GRIK2* being associated with disorders that have more wide ranging effects on the central nervous system.

8.4.2 Copy Number Analysis

The CNV analysis revealed many shared sites of CNV in the British family but none in the French family. Unfortunately the data quality for the CNV analysis was not ideal and so any identified CNVs would need further investigations to ensure they were genuine. It was deemed worthwhile to use QPCR to test for a possible CNV at the 6q16 disease region. The QPCR data and subsequent statistical analysis revealed no significant difference between the test samples and the control, indicating all had the same copy number and that it was normal. The genotyping data also suggested there was no CNV due to the presence of heterozygous calls across the region at which there was an apparent loss. If an allele were lost it would be evident in the genotyping data by the presence of consecutive homozygous calls.

Of the CNV analysis on the other chromosomes, just one site revealed a region of interest which, if genuine, would result in a loss of *ARID4A* in all British samples tested. This CNV was not indicated in the French family. *ARID4A* encodes AT-rich

interactive domain 4A on 14q23.1. This gene is also named retinoblastoma binding protein 1 (*RBP1*) and regulates cell proliferation by binding and inhibiting the activity of growth promoting protein, including the transcription factor E2F. However, though it is interesting that this CNV was consistent in all members of the British family analysed, this CNV is not in the linked disease region at 6q and nor was it seen in the French family. It is therefore unlikely to be significant in the disease state.

8.4.3 Summary

Genotyping analysis of members of a British PBCRA family and a French family with suspected PBCRA appears to have refined the disease region to between 6q16.1 and 6q16.2. The two families have a shared region of contiguous SNPs with common alleles that appear to segregate with the disease. Mutations in *SIM1* or the coding exons of *MCHR2* and *FBXL4* are not the cause of PBCRA. The primary candidate gene appears to be *GRIK2*.

9. SPLIT-HAND/SPLIT-FOOT MALFORMATION WITH NORTH CAROLINA MACULAR DYSTROPHY (SHFM & NCMD)

9.1 Genetic Analysis

This is a rare and unusual condition in which there is a macular phenotype that is consistent in penetrance but varies in appearance with additional split-hand/split-foot malformations that show varying penetrance. Loci for SHFM and images of the phenotype are shown in Section 2.5.5. In the two families being studied in this project (Figure 9.1), the disorder has not been linked to a specific locus. Analysis in this project has focused on regions of copy number variation identified by Dr A. Kalhoro during her own project. In the French family, a region of copy number decrease at chromosome 5p15.33 followed by an area of copy number increase at 5p15.32 was identified. The decrease was seen in five of six affected individuals analysed and in none of the three unaffected individuals whereas the increase in SNP copy number state was seen in all affected family members but not in the unaffected individuals. It is interesting to note that this overlaps with the MCDR3 disease locus (Section 6).

The English family showed no copy number variations in this region, which was surprising as it was expected that the two families would have a similar genetic basis. Dr A. Kalhoro identified a region of copy number increase in each of the three affected individuals was present at 6q27 that was not seen in the unaffected family member. A copy number variation in this region

of the genome is interesting as the homologue of the mouse *Brachyury T* gene is located in this area, mutations in which result in *abnormal feet and tail* in mice (Ruvinsky et al., 2002).

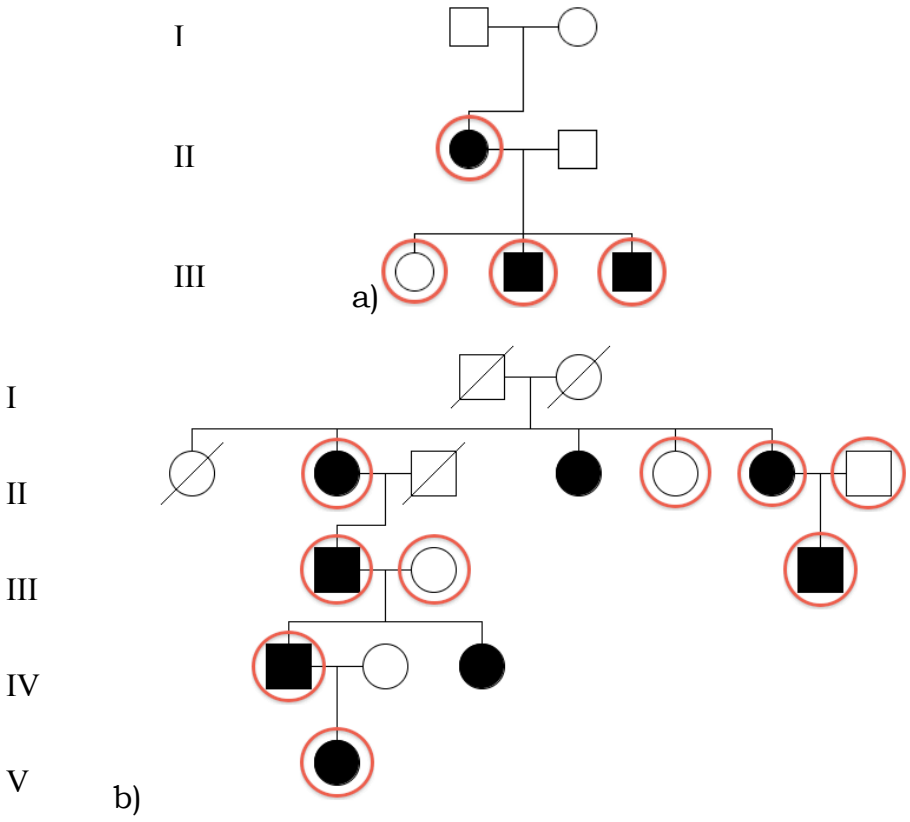


Figure 9.1 SHFM and NCMD pedigrees: a) a three-generation English family and b) a five-generation French family. Circled in red are samples run on the Affymetrix 250K SNP Chips.

9.2 Methods Applied

9.2.1 Candidate Screening

The English family members were screened for variations in the novel ncRNA AC026711.1 using the BIOTaq protocol and an annealing temperature of 60°C. The primers 5DelRNA (listed in Table 9.1) were used and the PCR product used directly in a sequencing reaction as the DNA template.

9.2.2 QPCR

QPCR experiments using SYBR Green were conducted to investigate copy number variations in affected individuals. The SYBR Green protocol was followed (Section 3.1.7). Primers for *GAPDH* were tested as in Section 8.2.3. The test primers are listed in Table 9.1. The 5DelRNA, 5DelE and 5DelF primers were tested for specificity with SYBR Green and all test sets produced a single amplicon. The QPCR plate contained three repeats for each sample and *GAPDH* control QPCRs were conducted on the same plate as one of the three test QPCRs. Three separate test QPCR regions were amplified within the suspected disease region. The 5DelF primers amplified 162bp overlapping the SNP rs1346474. 5DelE primers amplified 151bp overlapping the SNP rs261189. Finally, the primers 5DelRNA amplified a 179bp region containing the ncRNA AC026711.1 (4,302,889 – 4,302,974).

Table 9.1 Primers for QPCRs.

Primers	Name
TCCCACTGCTGTTGGGCTTCTCATGTT	5DelE F
AAGGATTTCTGTTTCCAAGGCTCAGTA	5DelE R
TTCCTCACCTTTCCTAGTTTTATGTG	5DelF F
CACGCATTCAAAGAGATGGGGCTTCCT	5DelF R
CAATTATAGAAAACCTCAACTTGGGAC	5DelRNA F
TGCCAGCGAGAACCCTGACAAACAGAC	5DelRNA R

9.3 Results

9.3.1 Affymetrix Data

Genotyping Analysis

For this analysis all members for whom DNA was available were processed over 250K *Sty I* Affymetrix SNP chips, obtaining genotype data for both affected and unaffected family members. The usefulness of this in identifying Mendel errors is apparent in

the analysis. When the genotyping data for the French family samples were aligned and Mendel checks carried out, a run of errors was identified in more than one sample over the same region on chromosome 5, suggesting loss of one allele over a number of SNPs. For example, patient III:1 is the affected parent of IV:1 and the unaffected parent is III:2 (Figure 9.1b). In Figure 9.2, there is a run of consecutive SNPs from rs7716544 (4,296,367) to rs17677148 (4,399,737) at which IV:1 is apparently homozygous for alleles which correspond only to the genotype of the unaffected parent, III:2. Over this range IV:1 should be heterozygous (AB) as III:1 is apparently homozygous for the opposite alleles to those of III:2. Similarly, III:1 appears to only have inherited alleles from his unaffected parent at the same region of SNPs, Figure 9.3.

III:1	III:2	IV:1	Mendel	dbSNP RS ID		
BB	BB	BB	MenOK	rs11134035		RP11-445O3.1
BB	AB	BB	MenOK	rs7715735		
BB	AB	BB	MenOK	rs1346474		
AA	AA	AA	MenOK	rs458537		
AA	AA	AA	MenOK	rs456741		
AA	BB	BB	Error_1	rs7716544		AC026711.1
BB	AA	AA	Error_1	rs261189		
BB	AA	AA	Error_1	rs261187		
AA	BB	BB	Error_1	rs261182		RP11-445O3.1
BB	AA	AA	Error_1	rs261181		
BB	AA	AA	Error_1	rs155037		
AA	BB	BB	Error_1	rs261166		
AA	AB	BB	Error_1	rs17677148		AC106799.1
BB	BB	BB	MenOK	rs1428984		RP11-445O3.2

SNPs that lie within gene regions

Figure 9.2 Excel sheet extract that shows the region of SNPs in which a run of Mendel errors was identified in IV:1 when compared to the affected parent (III:1) and unaffected parent (III:2).

II:2	Dummy II:3	III:1	Mendel	dbSNP RS ID		
BB	NoCall	BB	MenOK	rs11134035		RP11-445O3.1
BB	NoCall	BB	MenOK	rs7715735		
AA	NoCall	BB	Error_1	rs1346474		
AA	NoCall	AA	MenOK	rs458537		
AA	NoCall	AA	MenOK	rs456741		
BB	NoCall	AA	Error_1	rs7716544		AC026711.1
AA	NoCall	BB	Error_1	rs261189		
AA	NoCall	BB	Error_1	rs261187		
BB	NoCall	AA	Error_1	rs261182		RP11-445O3.1
AA	NoCall	BB	Error_1	rs261181		
AA	NoCall	BB	Error_1	rs155037		
BB	NoCall	AA	Error_1	rs261166		
AA	NoCall	AA	MenOK	rs17677148		AC106799.1
BB	NoCall	BB	MenOK	rs1428984		RP11-445O3.2

Figure 9.3 Excel sheet extract that shows the region of SNPs in which a run of Mendel errors was identified in III:1 when compared to the affected parent (II:2) and a dummy unaffected parent.

Individuals III:3 and V:1 have only a single error, each at one end of the apparent deletion region but over the other SNPs in the region only homozygous calls are seen (III:3 shown in Figure 9.4). For III:1 and III:3 the first error was detected at rs1346474 (4,220,728) but homozygous calls were given at many SNPs prior to this, suggesting the deletion may begin prior to this SNP.

II:6	II:7	III:3	Mendel	dbSNP RS ID		
BB	BB	BB	MenOK	rs11134035		RP11-445O3.1
BB	BB	BB	MenOK	rs7715735		
AA	BB	BB	Error_1	rs1346474		
AA	AB	AA	MenOK	rs458537		
AA	AB	AA	MenOK	rs456741		
BB	AB	BB	MenOK	rs7716544		AC026711.1
AA	AA	AA	MenOK	rs261189		
AA	AA	AA	MenOK	rs261187		
BB	BB	BB	MenOK	rs261182		RP11-445O3.1
AA	AA	AA	MenOK	rs261181		
AA	AA	AA	MenOK	rs155037		
BB	AB	BB	MenOK	rs261166		
AA	AA	AA	MenOK	rs17677148		AC106799.1
BB	BB	BB	MenOK	rs1428984		RP11-445O3.2

SNPs that lie within gene regions

Figure 9.4 Excel image showing a single Mendel error in III:3.

Though errors were detected between SNPs rs1346474 to rs17677148, heterozygous calls in affected samples were not seen over a wider area than this, whereas for the unaffected samples heterozygous calls were seen throughout the region. The heterozygous calls seen in affecteds closest to the telomeric end of the region of errors were seen at rs11134030 (4,065,252) and after

the end of the error region at rs11744458 (4,664,870), shown in Figure 9.5. This suggests the deletion may extend beyond where the error calls identify loss of alleles. Annotations in this region contain no known characterised genes but upstream lies *IRX1,* C5orf38 and *IRX2* and downstream is *ADAMTS16*. The deletion also overlaps the region of the MCDR3 marker D5S2088, therefore it is possible the two disease states may be caused by different changes in the same gene. Between the SNPs rs11134030 and rs11744458 are the annotations: AC025187.1, CTD-2008N3.1, RP11-445O3.1, AC026711.1, AC106799.1, RP11-445O3.2 and CTD-2318H23.1. None of these have been characterised. AC025187.1 and AC106799.1 are annotated as known protein coding genes. CTD-2008N3.1, RP11-445O3.1, RP11-445O3.2 and CTD-2318H23.1 are processed transcripts and AC026711.1 is a novel ncRNA.

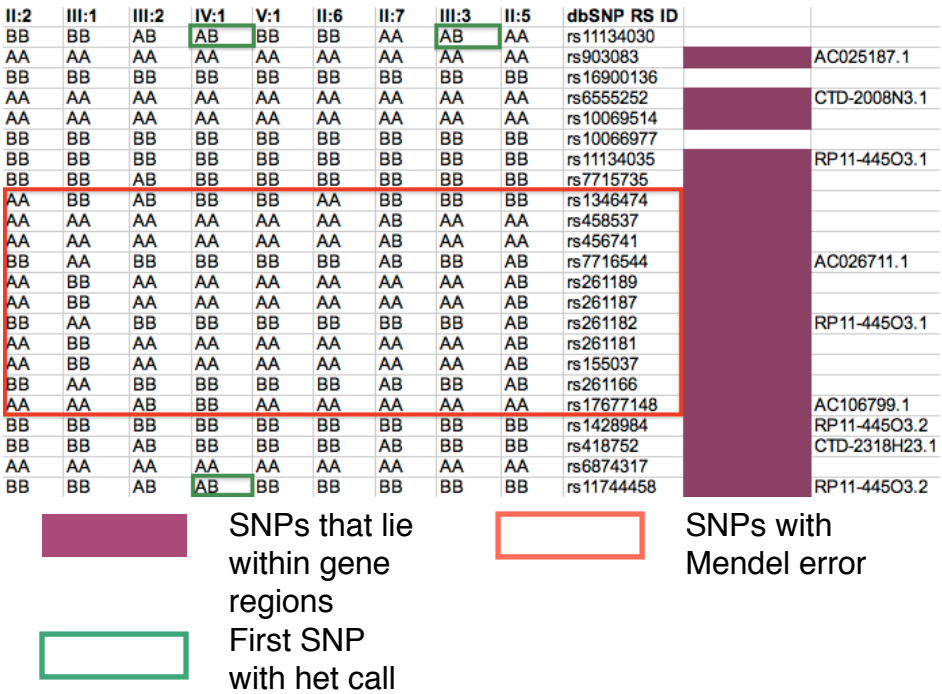


Figure 9.5 Excel image showing the genotype calls for subjects from the pedigree in Figure 9.1b. The region highlighted in red covers the region of errors highlighted by the Mendel checks. The green boxes highlight the first heterozygous calls seen in affected samples.

Looking at the English family SNPs in this region, the same errors in the pattern of inheritance were not seen. At this 5p region, the English family genotypes showed numerous heterozygous calls from rs7716544 (4,296,367) onwards. However, homozygous calls are seen before this point, therefore it is possible that a microdeletion is present in the same region where it was identified in the French family. From Figure 9.6 it is clear however that the genotypes of the unaffected offspring are very similar to the affecteds. The previous CNV analysis by Dr A. Kalhoro did not identify a deletion in this family at the 5p region and Figure 9.6 aligns the English family genotypes across the same SNPs as in Figures 9.2 - 9.4. The genotyping data appears to confirm that there is no deletion at this region in the English family.

II:1	Dummy II:2	III:1	III:2	III:3	dbSNP RS ID		
BB	NoCall	BB	BB	BB	rs11134035		RP11-445O3.1
BB	NoCall	BB	BB	BB	rs7715735		
BB	NoCall	BB	BB	BB	rs1346474		
AA	NoCall	AA	AA	AA	rs458537		
AA	NoCall	AA	AA	AA	rs456741		
AB	NoCall	AB	AB	AB	rs7716544		AC026711.1
AB	NoCall	NoCall	NoCall	BB	rs261189		
NoCall	NoCall	NoCall	AB	AA	rs261187		
AB	NoCall	NoCall	AB	NoCall	rs261182		RP11-445O3.1
AB	NoCall	AB	NoCall	BB	rs261181		
AB	NoCall	AB	AB	AB	rs155037		
AB	NoCall	AB	AB	AB	rs261166		
AB	NoCall	AB	AB	AB	rs17677148		AC106799.1
BB	NoCall	BB	BB	BB	rs1428984		RP11-445O3.2

Figure 9.6 Excel sheet showing the 5p genomic region as seen in Figures 9.2 - 9.4 but here the genotypes of the English family are shown and reveal no Mendel errors.

The Mendel errors seen in the French affected members are summarised in Table 9.2. The heterozygous calls found in the same region are also summarised as an indication of how large the actual deletion may be.

Table 9.2 Summary of the Mendel errors in the affected French family members. Heterozygous calls at the same region indicate how large the actual deletion may be.

	dbDNP RS ID	Position	Distance Between SNPs (bp)
First Mendel Error	rs1346474	4,220,728	179,009
Last Mendel Error	rs17677148	4,399,737	
First Heterozygous Call	rs11134030	4,065,252	599,618
Last Heterozygous Call	11744458	4,664,870	

Copy Number Analysis

The quality of the sample data was relatively poor for both families but despite this the analysis appeared to identify CNVs. In previous analysis, Dr A. Kalhoro identified common regions of CNV that required further investigation. A deletion was highlighted at 5p15.33 in all but one affected individual of the French family that was not seen in any of the unaffecteds or members of the English family. The deletion appeared to run from rs655652 (4,137,004) to rs261165 (4,340,479), which corresponds with the Mendel errors in the genotyping data (Figure 9.7).

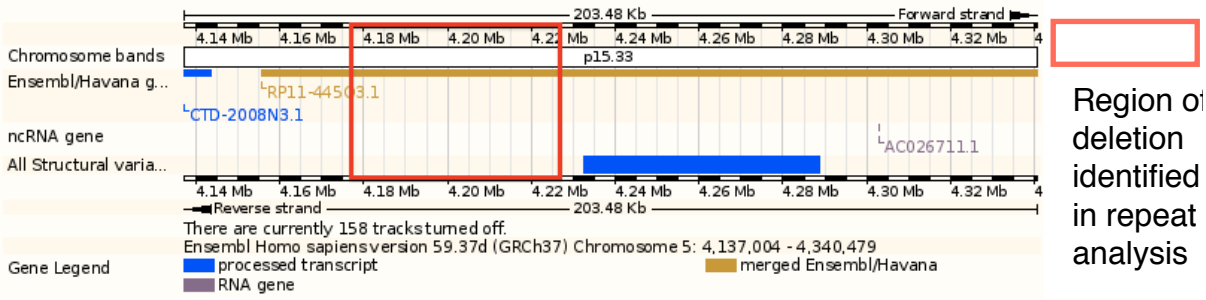


Figure 9.7 Ensembl image of the original region of deletion identified in the French family by Dr A. Kalhoro with the analysis highlighted by the new CNV analysis highlighted by the red box.

The repeat CNV analysis conducted with the new Genotyping Console in this project identified a CN state of 1 between rs11134035 (4,175,312, which suggests the deletion begins further upstream near the first heterozygous call in Figure 9.5) and rs1346474 (4,220,728) in three affected members of the French family (highlighted by the red box in Figure 9.7). A duplication was then identified in five affected members of the French family between rs17677148 (4,399,737) and rs1428984 (4,626,460), shown in Figure 9.8 in the red box.

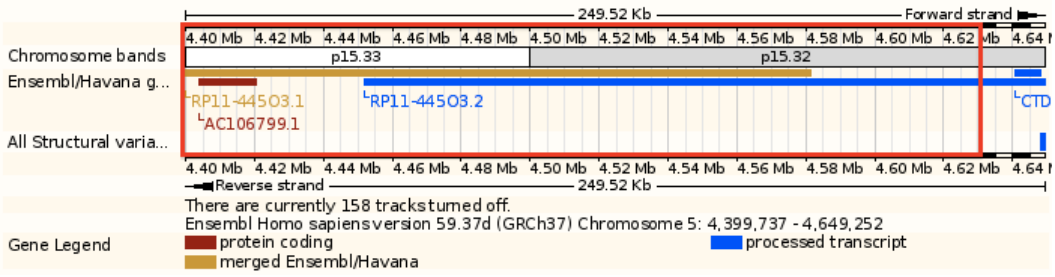


Figure 9.8 Ensembl image showing the overlap of the duplication region identified from Affymetrix 250K CNV analysis by Dr A. Kalhoro in the French family with the new analysis highlighted in the red box.

The DNA from III:3 was further analysed on a SNP 6.0 chip, which contains one million SNPs across the genome and is ideal for identifying CNVs. A CN state of 1 was given between the SNPs rs4479812 (4,216,057) and rs9942406 (4,409,489) and a CN state of 4 (triplication of one allele or double duplication) given between CNV sites CN_112820 (closest SNP rs9942406) to CN_1137043 (closest SNP rs293127, 4,622,584). These regions are shown in Figure 9.9, taken from the Chromosome Analysis Suite (CAS). The CAS uses the human genome build 36 as reference so the physical positions in Figure 9.9 are different from those used

elsewhere in this project, which are from the most recent human genome build, 37. In the white box of Figure 9.9, the red and blue bars at the top represent the region where a run of copy number loss (CN state of 1, red) was identified and appears to be immediately followed by a run of duplication (CN state of 4, blue). The log2 Ratio shows the trace of the raw data whilst the orange line above this depicts the CAS interpretation of the raw data. The region at which there are consecutive CN state calls of one extends over 167 markers whilst a CN state of 4 is given over 156 consecutive markers. The data from a control sample are also shown in Figure 9.9. This confirms the analysis of the 250K genotyping and CNV analysis.

The SNP 6.0 analysis also detected a loss of one allele at 1p33, resulting in a predicted deletion of an intron of *AGBL4*. A CN state of 4 was also detected at 17q11.2 that would result in a duplication of *CRLF3.* As only one family member had these regions highlighted, it is unlikely that these CNVs are significant. Neither of these genes are listed as being expressed in retina or RPE/choroid, and neither are at known SHFM or macular dystrophy loci.

The data returned from the SNP 6.0 array analysis of one sample confirmed the 250K data and indicated the deletion region to be ~200kb in size. A duplicated region with a gain of 4 directly following the deletion was detected, which also appeared to be ~200kb in size. This implies a duplication on both chromosomes or a triplication on one. There are a few annotations within both the deletion and the duplication regions and all are uncharacterised.

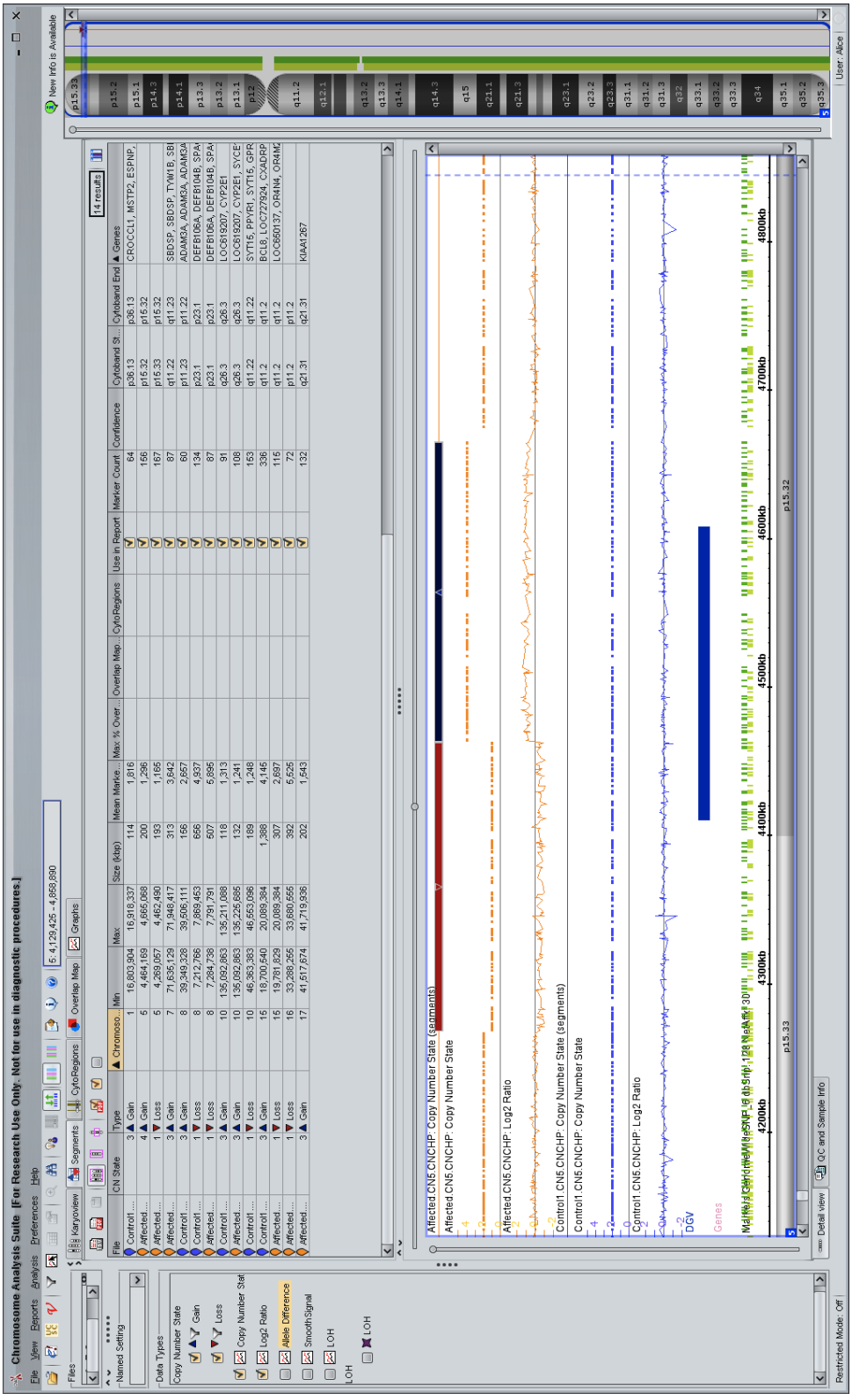


Figure 9.9 Data from the SNP 6.0 sample analysed in the Chromosome Analysis Suite highlighting the region of deletion followed by duplication on chromosome 5.

The deletion region contains the majority of exons of the known processed transcript RP11-445O3.1, a novel non-coding RNA AC026711.1 and the partial sequence of the known processed transcript RP11-445O3.2 (Figure 9.5). The duplication region contains only the partial sequence of this latter transcript (Figure 9.8). It is unclear whether the deletion or duplication is more likely to be involved with the disease state, although hemizygous loss of gene function would generally be more detrimental. However, gene dosage with either less or more than normal copies of genes is important in disease (Emanuel and Shaikh, 2001).

When these CNVs were first identified they appeared to be novel but subsequently other CNVs have been detected in normal populations. In the Ensembl genome build 37 there is an annotated region between 4,232,530 and 4,288,545 (which is before the ncRNA AC026711.1) and another between 4,648,027 to 4,661,512 (at CTD-2318H23.1) identified in the study by Itsara et al. (2009). The Chromosome Analysis Software used to interpret the SNP 6.0 data showed the deletion and duplication to overlap with a known CNV, evident in Figure 9.9 as a blue band at the bottom of the image. This represents the position of the CNV on the previous human genome build (36). The CAS links to the database for genomic variants (DGV) and highlights them in the analysis window. However, not all current known CNVs are presented in Figure 9.9 as the comparisons were made to the human genome build 36 database. The CNVs in the current DGV (build 37) that have been found within the 5p region are highlighted in this family and shown in Table 9.3. The French family data and known areas of CNV are summarised in Figure 9.10.

Table 9.3 Summary of the CNV regions identified in the French SHFM and NCMD samples in the various analysis types and compared to known CNVs.

Analysis Type	SNP	Loss/Gain	Physical Position	Known CNVs
SNP 6.0	rs4479812	first SNP with loss	4,216,057	Itsara et al. (2009): nsv461916 4,232,530 - 4,288,545 Conrad et al. (2009): DGV68994 4,325,180 - 4,325,851
	rs9942406	last SNP with loss and closest to gain	4,409,489	Wong et al. (2007): DGV4430 4,357,812 - 4,555,531 Conrad et al. (2009): DGV64098 4,465,031 - 4,466,632
	rs293127	last SNP closest to gain	4,622,584	Bentley et al. (2008): DGV46892 4,579,127 - 4,579,378
250K Genotyping	rs1346474	first SNP with loss	4,220,728	Itsara et al. (2009): nsv461916 4,232,530 - 4,288,545
	rs17677148	last SNP with loss	4,399,737	Conrad et al. (2009): DGV68994 4,325,180 - 4,325,851 Wong et al. (2007): DGV4430 4,357,812 - 4,555,531
250K CNV	rs11134035	first SNP with loss	4,175,312	
	rs1346474	last SNP with loss	4,220,728	
	rs17677148	first SNP with gain	4,399,737	Wong et al. (2007): DGV4430 4,357,812 - 4,555,531
	rs1428984	last SNP with gain	4,626,460	Conrad et al. (2009): DGV64098 4,465,031 - 4,466,632 Bentley et al. (2008): DGV46892 4,579,127 - 4,579,378
		first SNP with loss		
		last SNP with loss/ first of gain		
		last SNP with gain		

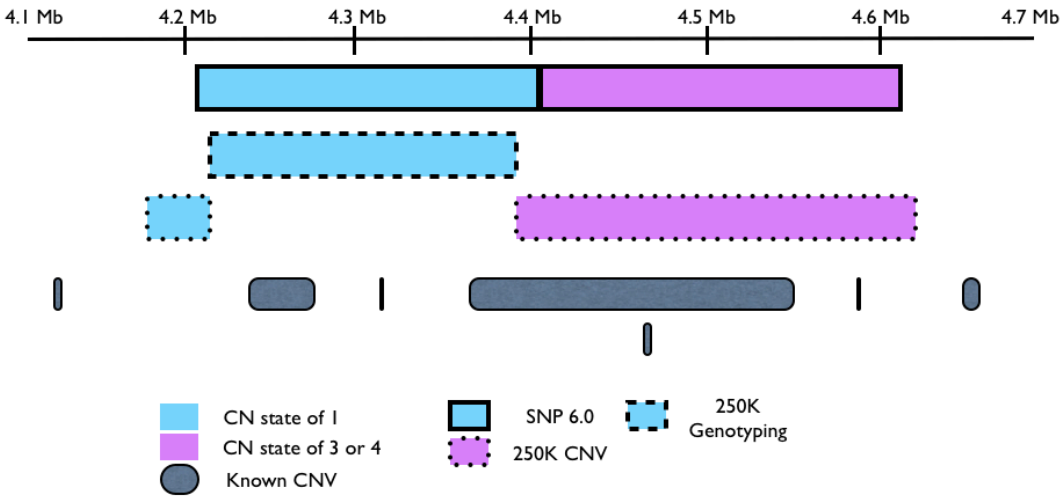


Figure 9.10 Image showing the relative position of the CNVs identified in the NCMD and SHFM analysis and the relative positions of the known CNVs described in Table 9.3.

Previous analysis by Dr A. Kalhoro revealed a duplication in affected members of the English family at 6q27. However, reviewing the CNV data has revealed the 6q27 CNV to be a known region of CNV and therefore is unlikely to be significant to the disease state. The 250K CNV analysis for the English family revealed numerous sites across the entire genome where the affected samples had common CNVs. These are shown in Table 9.4. Known regions of CNV (when compared to the DGV) have been excluded from this Table. None of the regions identified were in known SHFM loci and though there are numerous CNV regions containing genes, none stand out as primary candidates.

Table 9.4 CNVs common to all English family affected members and not present on the DGV.

Chromosome	Cytoband Start	Cytoband End	Gain/Loss	Genes within CNV
1	p36.23	p36.23	3	*RERE*, arginine-glutamic acid dipeptide (RE) repeats
	p36.21	p36.21	3	*CTRC*, chymotrypsin, *CELA2A & B*, chymotrypsin-like elastase family, member 2A, *CASP9*, caspase 9, apoptosis-related cysteine peptidase, *DNAJC16*, DnaJ (Hsp40) homolog, subfamily C, member 16, *AGMAT*, agmatine ureohydrolase, *DDI2*, DNA-damage inducible 1 homolog 2 (S. cerevisiae), *RSC1A1*, regulatory solute carrier protein, family 1, member 1, *PLEKHM2*, pleckstrin homology domain containing, family M (with RUN domain) member 2
	p31.3	p31.3	3	*PDE4B*, phosphodiesterase 4B, cAMP-specific
	p31.1	p31.1	1	*PTGFR*, prostaglandin F receptor
2	p23.1	p22.3	1	*KRT18P52*, keratin 18 pseudogene 52, *DPY30*, dpy-30 homolog (C. elegans), *MEMO1*, mediator of cell motility 1
	p14	p14	3	*CNRIP1*, cannabinoid receptor interacting protein 1, *PLEK*, plekstrin
	p12	p12	3	*LRRTM4*, leucine rich repeat transmembrane neuronal 4
	q21.3	q22.1	3	*CXCR4*, chemokine (C-X-C motif) receptor 4
3	p24.2	p24.2	3	*UBE2E1*, ubiquitin-conjugating enzyme E2E 1 (UBC4/5 homolog, yeast), *NKIRAS1*, NFKB inhibitor interacting Ras-like 1, *RPL15*, ribosomal protein L15, *NR1D2*, nuclear receptor subfamily 1, group D, member 2
	p14.3	p14.3	1	*ERC2*, ELKS/RAB6-interacting/CAST family member 2
4	p16.3	p16.3	3	*ZFYVE28*, zinc finger, FYVE domain containing 28, *RNF4*, ring finger protein 4
	q13.3	q13.3	3	*IL8*, interleukin 8, *CXCL1, 5 & 6*, chemokine (C-X-C motif) ligand, *PF4V1*, platelet factor 4 variant 1, PF4, platelet factor 4, *PPBP*, pro-platelet basic protein (chemokine (C-X-C motif) ligand 7)
	q22.3	q22.3	3	C4orf37
	q34.1	q34.1	1	*GALNTL6*, UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase-like 6
5	q31.1	q31.1	3	*FNIP1*, folliculin interacting protein 1, *RAPGEF6*, Rap guanine nucleotide exchange factor (GEF) 6
	q31.1	q31.1	3	*SLC22A5*, solute carrier family 22 (organic cation/carnitine transporter), member 5, *IRF1*, interferon regulatory factor 1, C5orf56
	q35.3	q35.3	3	*N4BP3*, NEDD4 binding protein 3, *RMND5B*, required for meiotic nuclear division 5 homolog B (S. cerevisiae), *NHP2*, NHP2 ribonucleoprotein homolog (yeast)
6	p12.1	p12.1	1	*DST*, dystonin
	q21	q21	3	*AMD1*, adenosylmethionine decarboxylase 1, *GTF3C6*, general transcription factor IIIC, polypeptide 6, alpha 35kDa, *RPF2*, ribosome production factor 2 homolog (S. cerevisiae)
	q25.2	q25.2	1	*TIAM2*, T-cell lymphoma invasion and metastasis 2
7	p13	p13	3	*NPC1L1*, NPC1 (Niemann-Pick disease, type C1, gene)-like 1, *DDX56*, DEAD (Asp-Glu-Ala-Asp) box polypeptide 56, *TMED4*, transmembrane emp24 protein transport domain containing 4, *OGDH*, oxoglutarate (alpha-ketoglutarate) dehydrogenase (lipamide)
8	p12	p12	1	*RBPMS*, RNA binding protein with multiple splicing
9	p13.3	p13.3	3	*CD72 - TMEM8B*
10	q21.3	q21.3	3	*RPL12P8*, ribosomal protein L12 pseudogene 8, *SIRT1*, sirtuin 1, *HERC4*, hect domain and RLD 4
12	q13.13	q13.13	3	*SMUG1*, single-stranded-selective monofunctional uracil-DNA glycosylase 1, *CBX5*, chromobox homolog 5, *HNRNPA1*, heterogenous nuclear ribonucleoprotein A1, *NFE2*, nuclear factor (erythroid-derived 2), *COPZ1*, coatomer protein complex, subunit zeta 1, *GPR84*, *ZNF385A*
	q23.3	q23.3	3	*CKAP4*, cytoskeleton-associated protein 4, *TCP11L2*, t-complex 11 (mouse)-like 2
14	q23.1	q23.2	3	*PRKCH*, protein kinase C, eta, *HIF1A*, hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)
15	q22.31	q22.31	3	*DPP8*, dipeptidyl-peptidase 8, *PTPLAD1*, protein tyrosine phosphatase-like A domain containing 1, *SLC24A1*, C15orf44, *DENND4A*, DENN/MADD domain containing 4A
	q23	q23	2	*IQCH*, IQ motif containing H
16	q24.1	q24.1	3	*KIAA0182*, *GINS2*, GINS complex subunit 2 (Psf2 homolog), C16orf74
17	q21.2	q21.31	3	*KCNH4 - CCR10*, small area in middle of known CNV : Jakobsson 2008
	q21.32	q21.32	3	*TBKBP1 - NFE2L1*
19	q13.32	q13.32	3	*ZNF541*, *GLTSCR1 & 2*, *EHD2*
20	q13.32	q13.32	3	*TH1L*, *CTSZ*, *TUBB1*, *ATP5E*, *SLMO2*
21	q21.3	q21.3	3	C21orf7, *BACH1*

9.3.2 QPCR

QPCRs were used to confirm the presence of a deletion in affected members of the French family, as determined by the Affymetrix genotyping and CNV analyses and to show the QPCR technique is able to detect such CNVs (previous PBCRA analysis contrasted to the Affymetrix CNV analysis to suggest no CNV was present). As for the previous PBCRA analysis the delta-delta Ct method was used to determine the CN state of each sample within the 5p genomic region. The CN state of sample genomes at the 5DelE, 5DelF and 5DelRNA regions are shown in Figures 9.11 – 9.13, respectively. 1 represents a normal CN state therefore a loss of one allele would provide a 0.5 output. Error bars represent the standard error of the mean (SEM). Comparison of the Control and unaffected sample III:2 for 5DelE revealed no significant difference ($p = 0.884$). The t-test comparisons of the Control with each affected sample are shown in Figure 9.11.

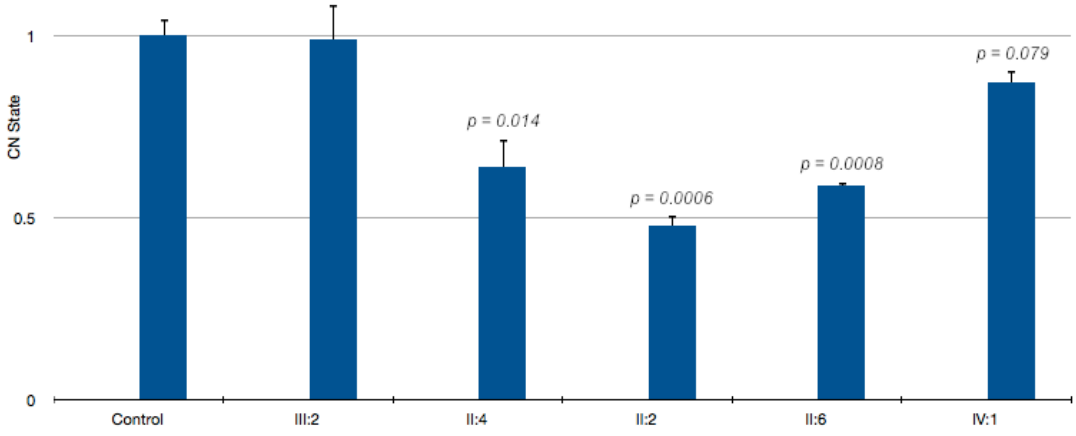


Figure 9.11 The CN state values over the 5DelE region calculated using the delta-delta-Ct method. III:2 is an unaffected member. Error bars represent SEM.

For the 5DelF region, ANOVA between the Control and unaffected III:2 was also not significant ($p = 0.258$). T-test comparisons with each affected sample are shown in Figure 9.12.

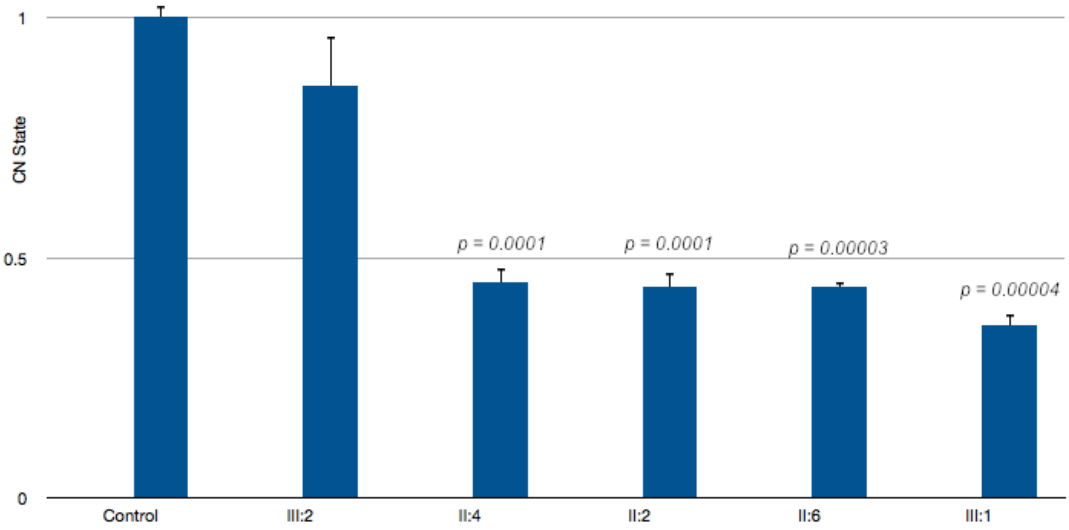


Figure 9.12 The CN state values over the 5DelF region calculated using the delta-delta-Ct method. III:2 is an unaffected member. Error bars represent SEM.

At the 5DelRNA region, ANOVA between the Control and unaffected samples II:5 and II:7 was slightly significant (p = 0.01), whereas the comparison with the affected samples was strongly significant (p = 0.000004). T-test comparisons with each affected sample are shown in Figure 9.13.

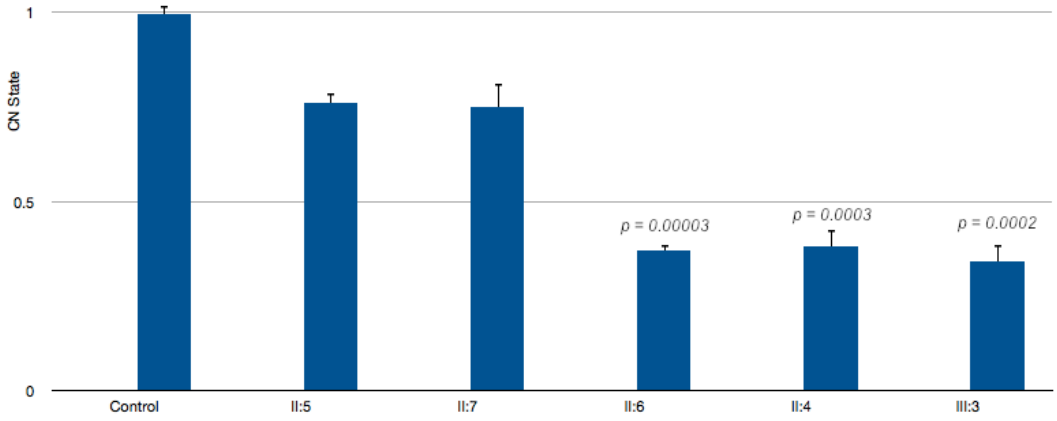


Figure 9.13 The CN state values over the 5DelRNA region calculated using the delta-delta-Ct method. III:2 and II:7 are unaffected members. Error bars represent SEM.

It is clear from the QPCR data that the affected samples shown contain a loss of DNA at the three regions tested, which each occur within the region detected in the genotyping and copy number analysis. Not only do the affected samples consistently show significantly less DNA than the control sample, but the unaffected samples consistently show no or minimal significant difference in comparison. The exception is for IV:1 at the 5DelE region. Though the genotyping and CNV data suggest a deletion at this region, the QPCR data is inconclusive. Whereas the other samples clearly show a significant reduction in the amount of DNA compared to the control and unaffected samples, IV:1 does not. This is likely to be due to an error in providing the same concentration of DNA for all samples. For samples of poor DNA quality, obtaining an accurate concentration, particularly when so little is used, will likely achieve errors. The standard error bars are small suggesting that within the experiment the data were consistent but ideally the analysis would be repeated to check the results and over a greater 5p region to find the limits of the CNV. Overall, Figures 9.11 – 9.13 confirm the French family affecteds carry a genome deletion at 5p15.33 not seen in the unaffected members, which includes loss of one copy of a novel ncRNA, AC026711.1.

Genomic DNA from one affected member of the English family (II:2) was used analyse CNV at the 5DelRNA region but a normal CN state was achieved.

Running a BLAST search with the predicted mature miRNA sequence of AC026711.1 (expanded in Figure 9.14) so as to identify genes that show complementarity within 3' UTRs, may reveal possible gene targets. This is not a precise method of predicting ncRNA targets as other factors are important to

consider in target prediction, such as thermostability and accessibility of the target binding site (Hammell, 2010). It does however, provide some idea of possible targets.

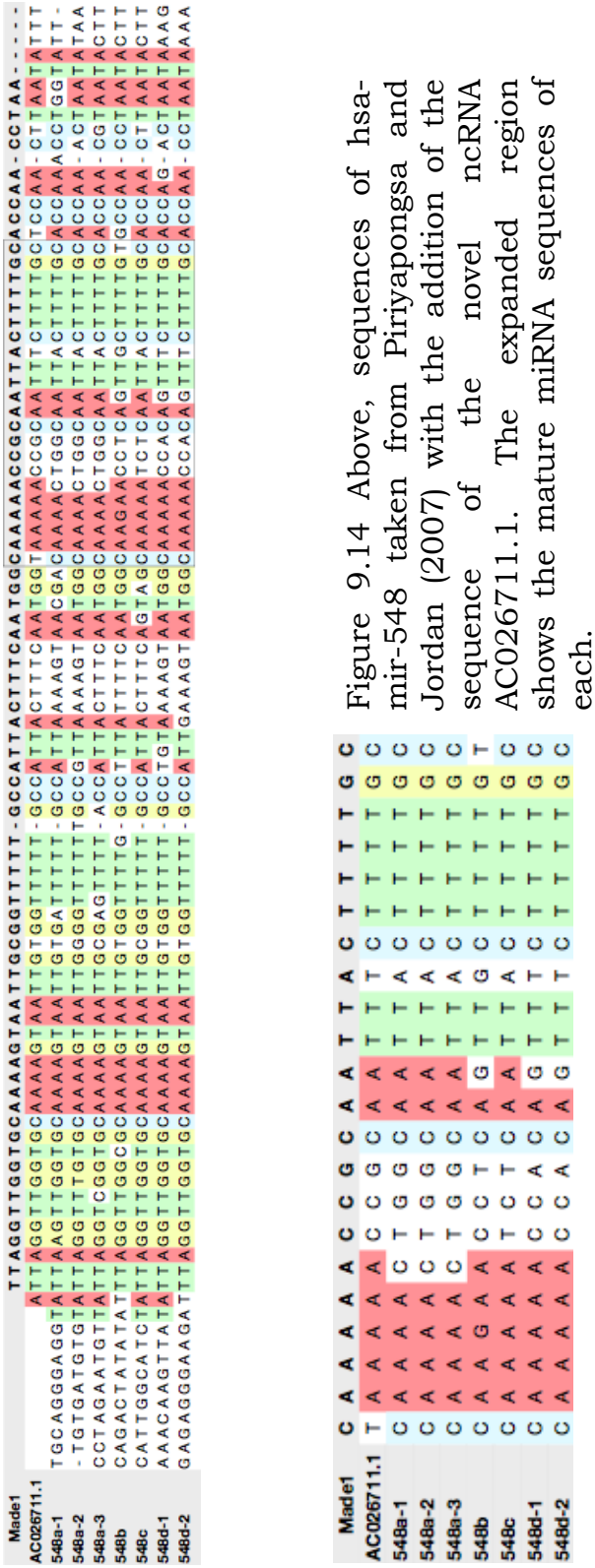


Figure 9.14 Above, sequences of hsa-mir-548 taken from Piriyapongsa and Jordan (2007) with the addition of the sequence of the novel ncRNA AC026711.1. The expanded region shows the mature miRNA sequences of each.

A BLAST search with the mature miRNA sequence revealed a number of genes with complementarity in 3' UTRs, which are shown in Table 9.5.

Table 9.5 Genes for which the predicted mature sequence of AC026711.1 showed complementarity to the 3' UTR sequences.

Gene	Name	Expression Profile
LRRC55	leucine rich repeat containing 55	Not listed in NEIBank retina or RPE/choroid databases but related members are
MTMR6	myotubularin related protein 6	RPE/choroid expression and related member *MTMR9* expression in retina. As MTMR6 and MTMR9 form heteromer which increases activity and stability of MTMR6 (Zou et al., 2009), it is possible *MTMR6* is also expressed in retina
CLEC7A	C-type lectin domain family 7	No recorded retina or RPE/choroid expression
TTC12	tetratricopeptide repeat protein 12	Not listed in NEIBank retina or RPE/choroid databases but related members are. *TTC12* cDNA listed in Unigene as detected in eye tissue. *TTC5* candidate for MCDR4
KATNAL1	katanin p60 subunit A-like 1	Not listed in NEIBank retina or RPE/choroid databases (see Discussion for more information)
SMU1	suppressor of mec-8 and unc-52 homolog (*C. elegans*)	Expression in both retina and RPE/choroid (see Discussion for more information)

9.4 Discussion

9.4.1 Genotyping Analysis

Analysis of two families with a Sorsby syndrome-like phenotype had previously been initiated by Dr A. Kalhoro and has been extended here. The analysis has highlighted regions of apparent error in Mendelian inheritance in a French family, identified as runs of homozygous calls in offspring over SNPs that should have been heterozygous based on the parent genotypes. In samples where parent data were not informative in Mendel checks, runs of homozygous calls were seen across the same group of SNPs, suggesting that a region of hemizygosity in the samples was also possible. Unaffected samples revealed heterozygous calls throughout the region, indicating no loss of allele. The errors identified in the Mendel check ran between rs1346474 (4,220,728)

and rs17677148 (4,399,737) but homozygous calls extended beyond these SNPs, suggesting the deletion region may extend beyond these points. The data were consistent with Dr A. Kalhoro's previous CNV analysis and the region lies within the MCDR3 locus. This analysis has highlighted the usefulness of analysing all members of a pedigree, both affected and unaffected. Without a disease locus, further genotyping analysis would reveal little informative analysis. The Mendel errors determined in the French family were not present in the British family.

9.4.2 Copy Number Analysis

SNPs with a CN state of 1 were identified between rs11134035 (4,175,312) and rs1346474 (4,220,728) in three of the affected samples from the French family but not in any of the unaffecteds or any of the English family members. A run of SNPs with a CN state of 3 were also detected from rs17677148 (4,399,737) to rs1428984 (4,626,460) in five of the affected samples. These results match the genotyping analysis but do miss a section of the deletion detected through the former analysis. The CNV analysis also failed to detect a deletion and duplication in all the affected samples, which may be explained by the poor sample quality. The deletion was shown by QPCR to be present in affected samples. Therefore it seems most likely that the reduced quality of the data for the Affymetrix CNV analysis was the reason for failing to detect a CNV. It would appear that there is a deletion in all of the French affected members that segregates with the disease state.

A single sample (III:3) was additionally analysed over a SNP 6.0 chip, an array containing one million SNPs and ideal for detecting CNVs. This analysis again identified a run of SNPs (167 consecutive) that revealed a CN state of 1 between rs4479812 (4,216,057) and rs9942406 (4,409,489). This was interesting

because the deletion had previously been inferred in this patient from the genotyping analysis in which just one SNP revealed a Mendel error with the remaining calls homozygous. The SNP 6.0 data was able to confirm the suspicion that this individual did also have a deletion across 5p15.33. The SNP 6.0 analysis also revealed a run of SNPs (156 consecutive) with a CN state of 4 directly following the deletion region and the gain of alleles extended to rs293127 (4,622,584). A CN state of 4 suggested there was either a triplication of one allele or a duplication of both alleles across this region. In the 250K CNV analysis however, samples were revealed to have a CN state of 3. As the data quality from the 250K arrays were somewhat unreliable, it is suspected that the CN state of 4 given by the SNP 6.0 array is the more accurate CN state call. This implies that the other four samples that gave a CN state of 3 from the 250K analysis may more accurately have a CN state of 4. Overall, the genotyping, CNV and QPCR reveal a deletion followed by a duplication of similar lengths on chromosome 5p15.33 that segregate with the disease state in the French family.

The SNP 6.0 analysis also revealed the single affected analysed had a deletion within an intron of *AGBL4* on 1p33 and a partial triplication of *CRLF3* on 17q11.2. *AGBL4* encodes ATP/GTP binding protein-like 4 and *CRLF3* cytokine receptor-like factor 3. Neither are listed on the NEIBank database of retina and RPE/choroid genes and do not seem likely candidates for the disorder. Given that just one sample was anlaysed on the SNP 6.0 array and that these CNVs were not detected in other members of the family in the 250K analysis, it seems more likely that these variations are unique to this individual.

9.4.3 Candidate Genes

Within the deletion and duplication regions are few annotations, all of which are uncharacterised. Predicted exons for the processed transcript RP11-445O3.1 and the novel ncRNA AC026711.1 are annotated in the region of deletion. Exons of RP11-445O3.1 extend into the duplication region although Ensembl does not describe a protein product for this annotation. The duplication also contains predicted exons of the processed transcript RP11-445O3.2 and the protein-coding gene AC106799.1. Here the ncRNA has been investigated due to the novel influence it may have.

In contrast to siRNAs, miRNAs tend to be distinct from annotated genes and have a distinct expression in cell types. They are processed from longer ncRNA structures that bind to partially complementary sequences on target mRNAs in the 3' untranslated regions (UTRs) and regulate expression by mRNA degradation and/or translational repression (Bartel, 2004). The micro RNA database (miRBase) has currently identified 1,048 human miRNA genes in the human genome build 37. In this project, Affymetrix and QPCR analysis have confirmed loss of one copy of the novel ncRNA AC026711.1 in affected members of the French family. This ncRNA was predicted from Rfam (ncRNA families) and miRBase databases. A sequence search in Rfam (Gardner et al., 2009) revealed sequence homology with the mir-548 family of miRNAs (Figure 9.14). A group of hsa-mir-548 sequences were identified by Piriyapongsa et al. (2007), who provided evidence showing these miRNAs are derived from transposable elements (TEs). They found seven closely related hsa-mir-548 sequences that co-localised with dispersed members of a single family of TEs known as Made1. These sequences are included in Figure 9.14

and the similarity of AC026711.1 with these sequences is apparent.

Though miRNAs can bind the 5' end or cleave within mRNA, the most common mechanism for preventing translation is by binding to the 3' UTR. A search for complementary sequences in genes using the predicted mature miRNA sequence from Figure 9.14 was run and produced many hits within the human genome, with 55 of them associated with characterised genes. Of these just six showed complementarity to the predicted AC026711.1 mature miRNA at the 3' UTR: *LRRC55, MTMR6, KATNAL1, TTC12, SMU1* and *CLEC7A* (Table 9.5). Of these only two appear to have interesting roles: *KATNAL1,* expression of which is needed for dendrite severing in *Drosophila* and katanin p60 has recently been shown to be required for skeletal re-organisation. The other interesting gene is *SMU1*, which suppresses *HSPG* expression (heparan sulphate proteoglycan core protein). The protein product of *HSPG* appears to have a role in the retina (Walz et al., 1997).

KATNAL1 encodes katanin p60 subunit A-like 1, which has been shown to be required for dendrite severing in *Drosophila* (Lee et al., 2009). Excess katanin p60 (KATNA1) results in short microtubules (Yu et al., 2008) and recently this microtubule-severing protein has been shown to be needed for skeletal reorganisation in mice (Iwaya et al., 2010). As *KATNAL1* has sequence similarities to *KATNA1* it is possible that the protein product also has similar functions. However, no expression in either the retina or RPE/choroid is listed in NEIBank databases.

TTC12 is a tetratricopeptide repeat protein, which again is not listed in retina or RPE/choroid databases but related members are. *TTC12* cDNA is listed in Unigene as detected in eye tissue so

there may be an as yet unidentified role for the protein in the eye. The related member *TTC5* has been identified in this project as a candidate for MCDR4.

SMU1 expression is listed in both retina and RPE/choroid databases and this encodes suppressor of mec-8 and unc-52 homolog (*Caenorhabditis elegans*). Interestingly, unc-119 homolog (*C. elegans*) mutations have been found to cause autosomal dominant CORD (Kobayashi et al., 2000; Section 2.3). The protein product of unc-52 is also known as perlecan, and the human ortholog is the basement membrane-specific heparan sulphate proteoglycan core protein (HSPG). This is closely related to the *Drosophila* eyes shut gene (eys, Husain et al., 2006) and mutations in the human *EYS* gene cause autosomal recessive RP (Abd El-Aziz et al., 2008). Expression of *HSPG* was shown by Regatieri et al. (2010) to parallel areas of choroidal neovascularisation in rat retina but in diabetic rats *HSPG* expression in the retina was decreased when compared to expression in the retina of normal rats (Bollineni et al., 1997). If *SMU1* suppresses *HSPG* and AC026711.1 suppresses *SMU1*, then with one less copy of the ncRNA there would be less suppression of *SMU1* and therefore more available to suppress to *HSPG,* resulting in a decrease of HSPG. If a decrease in HSPG is important in the phenotype of the retina in diabetic mice, then it may have an effect on the human retina as well.

9.4.4 Overlap with Known CNVs

Within the region of CNV identified in the affected members of the French family were a number of known CNVs (Table 9.3 and Figure 9.10). Of these, the biggest overlap came from the CNVs identified by Wong et al. (2007), which overlap with most of the region of duplication seen in the affected members of the French

family. The CNVs detected at this region by Wong et al. (2007) consisted of 11 gains and 12 losses from 95 control samples, so ~22% of control samples had a CNV. However, the region does not cover the region of deletion seen in all affecteds of the French family, suggesting that though the region of duplication may not be an uncommon CNV, the deletion may be unique. Itsara et al. (2009) published data identifying large CNV hotspots prone to recurrent mutation. They identified a CNV that sits within the region of deletion of the French family but prior to the ncRNA AC026711.1. From the publication data it appeared that the CNV identified at this site in the cohort was a duplication rather than a deletion. Though CNVs have been identified in the 5p15.33 region, the combination of a deletion followed by a duplication is an unusual occurrence so to find it consistently in every affected member of the French family suggests it may be significant.

The deletion and duplication appear to segregate with the disease in the French family and seem to be approximately the same size, which suggests that they may have arisen from a single event. In affected individuals, a region with a CN state of 1 was followed by a region with a CN state of at least 3 but possibly 4. This is a rare occurrence, although, variation at the site of duplication has been seen in the control population (Conrad et al, 2009 and Bentley et a., 2008). If this site may be duplicated irrespective of disease state then this may explain the CN state of 4 identified from the SNP 6.0 array data of subject III:3. This suggests an affected member may have a CN state of 3 or 4 depending on whether the individual also carries a normal CNV.

Given the similarities in the size of the deletion and duplication and that the deletion only seems to occur in the affected members, a duplication on the strand carrying the deletion may

be related to the deletion event, which may in turn be associated with the disease state. Though hemizygosity is thought to be more likely associated with a disease state, it is possible that the duplication is important. For example, though in a control population a small number of individuals were found to have a duplication, it may be that a single duplication is not enough to cause a physiological change but any levels above that do have an effect. Gene dosage is identified as an important factor in disease states and it could be that whist a CN state of 3 is not important, a CN state of 4 at this region is. For example, Emanueal and Shaikh (2001) have shown the importance of segmental aneusomy in disease, i.e. disorders that result from inappropriate gene dosage.

9.4.5 English Family Affymetrix Data

As this family is so small, linkage analysis has not been conducted and therefore the disease locus is not known. This means that unless the genotyping data produced regions of Mendel error, as occurred in the French family, the genotyping data would not be very informative.

Analysis at the 5p15.33 region did not reveal the same Mendel errors or runs of homozygous calls that were evident in the French family (Figure 9.6). The calls of the unaffected offspring analysed were very similar to those of the affecteds and hetereozygous calls were given for all samples analysed, indicating a region of deletion in these samples was unlikely. Without genotyping data for more family members, linkage analysis across the genome using the genotyping data is not possible. Attempts to analyse the chromosomes from the genotyping data would be uninformative due to the lack of meioses between samples.

Through the CNV analysis many sites of variation at which all affected samples exhibited common CNVs were identified (Table 9.4). The previous CNV at 6q27 identified by Dr A. Kalhoro was removed as a region of interest in this analysis due to the overlap with numerous known sites of CNV. Of the CNVs not listed in the DGV and common to the affected English family members many contained genes but just two currently appear to be of interest, *CXCR4* and *SIRT1*. A gain on 2q22.1 of *CXCR4* is interesting because this gene has been shown to be important in ocular neovascularisation (Lima e Silva et al., 2007) and to mediate photoreceptor tip cell morphology and vascular patterning in neonatal retina (Strasser et a., 2010). However, no roles have been described that would relate to the SHFM phenotype.

SIRT1 is involved in many pathways but it is particularly known for its role in repairing double-stranded DNA breaks. It is expressed throughout the retina and appears to be important in protecting cells from apoptosis though it is lack of this gene that causes problems (Ozawa et al., 2010). A gain of this gene was identified at 10q21.3 in the English family but what affect an extra copy may have in the retina or in development of limbs is unknown.

9.4.6 Summary

The affected members of the French family appear to have a region of deletion at 5p15.33 followed directly by a region of duplication of similar length. This lies within the MCDR3 locus and appears to segregate with disease as it was not seen in unaffected members of the family. There are no characterised genes within this region and it is suggested that loss of one copy of a novel predicted ncRNA could be influencing the disease state. The affected members of the English family did not show a CNV at

5p15.33 but did reveal numerous other regions of CNV at other areas of the genome.

10. BULL'S-EYE MACULAR DYSTROPHY (MCDR2)

10.1 Genetic Analysis

In the study by Michaelides et al. (2003b), the disease was linked to 4p15-p16.3, and *PROM1* identified as a possible candidate gene. This gene encodes human prominin (mouse)-like-1, a member of the prominin family of 5-transmembrane domain proteins and is expressed in retinoblastoma cell lines and adult retina (Jászai et al., 2007). The product of the mouse orthologue is concentrated in membrane evaginations at the base of the outer segments of rod photoreceptors. Mutations in this gene have already been found to cause retinal disease, such as the homozygous mutation in exon 15 (c.1,726C>T) identified in an Indian pedigree with an autosomal recessive retinal dystrophy (Zhang et al., 2007). This mutation results in formation of a truncated protein that was shown by functional studies in CHO cells not to reach the cell surface. A deletion in *PROM1* exon 16 (c.1,841delG) that causes a frameshift mutation at codon 614 and results in predicted premature termination of translation has also been shown to cause retinal degeneration (Maw et al., 2000). STGD4 has been linked to this locus (Kniazeva et al., 1999) and more recently Permanyer et al. (2010) have identified the mutation c.869delG in exon 8 as another cause of autosomal recessive RP. MCDR2 sufferers have been reported to show a substitution in exon 10 of *PROM1* (c.1,117C>T), which results in an amino acid change of arginine to cysteine (Arg373Cys, Figure 10.1; Michaelides et al., 2003a). Yang et al. (2008) developed a mouse model expressing this mutation in a human *PROM1* gene and demonstrated that the mutated protein disrupts photoreceptor

morphogenesis in a transgenic mouse model. The mutation caused effects predominantly in cones with sub-retinal deposits and photoreceptor atrophy characteristic of Stargardt disease evident. In their report, Yang et al. also stated that the screening of STGD4 and cone-rod dystrophy sufferers revealed this same *PROM1* missense mutation. The families did, however, have distinct disease haplotypes, indicating the mutations arose independently. Michaelides et al. (2010) re-assessed the STGD4, MCDR2 and RP families with the Arg373Cys mutation in addition to reporting two new British MCDR2 families in which the Arg373Cys mutation was identified in this project. Previous MCDR2 families have been linked with the marker D4S1601 with two different alleles identified. New families in this project will be screened to discover if they carry one of these alleles at this marker. If the same allele is identified that will indicate the families share the same ancestral mutation.

Another variation identified in *PROM1* that leads to a retinal dystrophy is the insertion c.1,349insT in exon 12. This mutation was identified in a CORD family and leads to a frameshift mutation at codon 452 and a premature stop codon at 464 (Pras et al., 2009). Mutations in *RIMS1* have also been shown to cause CORD (Johnson et al., 2003, Section 2.3). *RIMS1* encodes regulatory synaptic membrane exocytosis 1, which is expressed in the photoreceptors and thought to be important in synaptic transmission (Wang et al., 1997). It is possible that mutations in this gene may also arise in some members of the panels being analysed in this project.

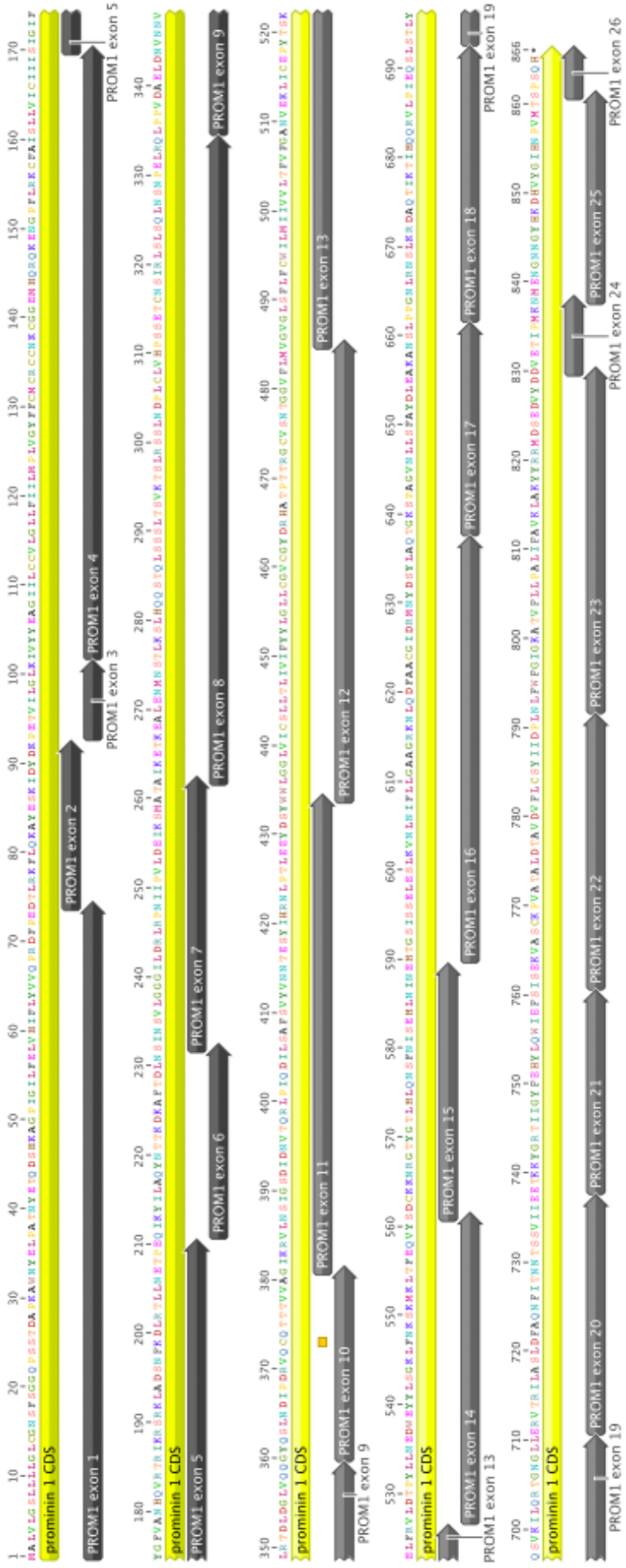


Figure 10.1 Amino acid sequence of PROM1 with the Arg373Cys mutation highlighted.

10.2 Methods Applied

10.2.1 Screening

RIMS1

Exon 14 of this gene was screened using the BioTaq PCR method (Section 3.1.1) in two suspected MCDR2 patients. These subjects did not have the *PROM1* exon 10 mutation and given their phenotype, it was considered a possibility that a mutation in *RIMS1* could be responsible for their disease state.

Table 10.1 Primers used for amplifying exon 14 of *RIMS1*.

Primers	Name
GACTTTCCATGAGTTATTAG	RIMS1 14F
TAGATCATTACAGCATAAT	RIMS1 14R

PROM1

Coding exons of *PROM1* were screened using the BIOTaq PCR method (Section 3.1.1). Primers were designed by Dr Richard Yang though some exons required new primers to be designed (Table 10.3).

10.2.2 Genotyping

For the genotyping of D4S1601, see Section 3.1.9. The primers used are listed in Table 10.2 and the reactions were carried out using a 56^{o}C annealing temperature.

Table 10.2 Primers used for amplifying D4S1601.

Primers	Name
AACTGGCTTCCCCACC	D4S1601 F
TCGGGCTACTTTTGGCTA	D4S1601 R

Table 10.3 Primers and annealing temperatures of the *PROM1* exons.

Primers	Name	Annealing Temperature (°C)
AAGGCTTCCAGAAGCTCTGAGGCA	0047 (1F)	55
GTGGGTGCGTTTGGAGATAAATCC	0048 (1R)	
GATCTTTTTCTAAATATGCATT	0051 (2F)	56
CAGCCAAAATTTTTCTCATACT	0052 (2R)	
TCTGCCAAAATTCCTCACCTGCGT	0053 (3F)	60
GAAAGGCTTTCCAAGAGCAACTTG	0054 (3R)	
TTGTGCTACAATATGTGCTGTTTC	0049 (4F)	56
CAGCCTAAAACACAATCAGTTGTT	0050 (4R)	
GCTCTTTCTTCTCTCTGCCTTTTCCTG	1a (5F)	56
ATCGCGGTACATAGAGATGATGG	1b (5R)	
GCCCTCTTATTGCCTTTGGACC	2a (6F)	56
GTCTTCCCCCAACTTTCACG	2b (6R)	
AACTCTGGTCTAGCCGAAGAGGTTA	A7F	60, gel-extracted
GACCTGAACTGTCTGCATGGCCACG	A7R	
GCAGCTGTTAGAGCAGCCTAGCGATG	NEW 8F	60, gel-extracted
CTAACTGTTCTCCAAGTCAGGCCAC	NEW 8R	
CTGCGATTGTACCCTGTAG	9F	56
GCTATCACCCTTCTTGGCAACC	9R	
GCCTCTCTACTCGTACTG	10F	55, gel-extracted
GCAATACTTGGCAACACATTTCTC	10R	
GGTGGTTCAGGCTTTTCTGTTTGG	11F	56
CACCAGCTCCAAGGAGACAC	11R	
GAATAACTCATCGGCTCCAGCCTTAGTC	A12F	60, gel-extracted
GTTCATGTAACACCAGAGTCAGCACCCAGTC	A12R	
ATGATCTGTTTCACCTGAATAACACTGAAG	A13F	60, gel-extracted
CTGCTTATAAGTTTGCACTGCTCT	A13R	
GTATGTATGTTAGTGTAAAACTG	14F	58, gel-extracted
CTCATTCCAGAAAAAGAACATC	14R	
CCTTTCTCCTCATCTTCAGTGG	15F	56, gel-extracted
CCTAGATTTGGTGAAGGAATGTG	15R	
GGCATGAGCCACCACATCCAGC	16F	56
CCTAAAGGATCAAGCATGAACAC	16R	
GCCTACTAGATGTTGTGTTAAGGC	17F	56, gel-extracted
GGAACCTCTCCAGCAGC	17R	
GGATAGCGAGAGTGCTTTGAG	18F	58, gel-extracted
GCTGGGACCTATGAGAGATGAGC	18R	
GATTTGATGGCTATCTTGTGGGAAG	NEW 19F	58
GTCTGCACTTAGAGAAGTACTTG	NEW 19R	
AGCTGAGTTACAGTACTTGAATCA	NEW 20F	58
GAAGTCTTGGTCCTGCACATCAATG	NEW 21R	
GGAGATCCTTTTTGTGACACC	22F	58
GGTTTTGGATTCTCTCAAGCAG	22R	
CTTCACACAGTGCCTGGTCC	23F	58
CTTTGAAGACAGCACCACC	23R	
GGGGGATGTAGTTGCTGAGC	24F	58
CCTCCCCCATCCCATCTAGG	24R	
GTAGTCCTTTGGTCTTTGAAG	25F	58
CATACAGAGAGAAGTGAAGGC	25R	
GCTTCTTAGCACAGAGGTGATG	26F	58, gel-extracted
CAGAGGGTGGACTGGAC	26R	
TTCCTTAGTTCTTAGGCTCCTGGT	27AF	58
CATCTAAGTTGTGAGTCGCTGGATC	27CR	

10.3 Results

10.3.1 MCDR2 Panel

PROM1

This panel consisted of twenty-one subjects diagnosed with BEM and suspected MCDR2 with an additional unaffected parent of three of these samples analysed. Only half the samples had all 27 exons screened for variations, this is because the original panel consisted of only 11 samples and for the duration of the project samples would arrive for screening one at a time and so these were screened for the exon 10 mutation only.

Of the samples screened, three new MCDR2 families with a c.1,117C>T mutation encoding an Arg373Cys substitution were identified (Figure 10.2); two of these families have been reported in Michaelides et al. (2010).

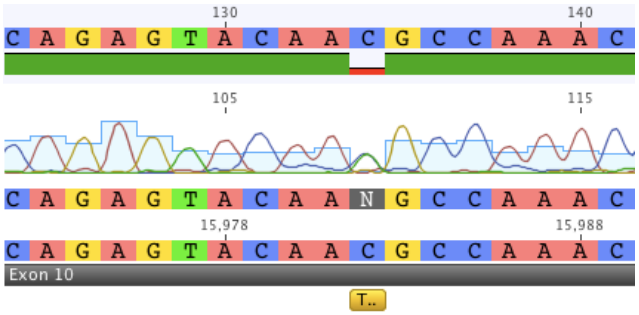


Figure 10.2 Image showing heterozygosity in exon 10 at position 1,117 for a C>T mutation in the third new MCDR2 family identified.

In the full *PROM1* screen, a handful of variations in nucleotide sequence were identified (Table 10.4). Five samples had the low frequency allele at rs3815344, which lies in intron 21; two families revealed a low frequency allele at rs2286455 in exon 8 and all affecteds had A and G nucleotides at rs6449209 upstream of exon 23 (Figure 10.3a). For one family A/A was seen at rs6449209 in addition to an inserted G prior to the SNP (Figure 10.3b). This insertion is not described in the NCBI SNP database.

All samples had the 42bp rs33950047 insertion within intron 20. One sample had a variation in exon 19 not seen in her two affected siblings, this variation was not annotated in Ensembl and does not alter the amino acid sequence.

Table 10.4 Nucleotide variations in samples screened for mutations in *PROM1*.

PROM1 Variation	Allele Frequency	Variation	Exon/Intron	Variations
rs2286455	A = 0.112 G = 0.888	A/G	Exon 8	A/G in two siblings (family 1), A/A in mother and son (family 2)
		C/T	Exon 19	T/T at nucleotide 2,112 in one sibling of family 3, synonymous change
rs33950047	No frequency data	Insertion of 42bp	Intron 20	Insertion in all samples
rs3815344	C = 0.923 G = 0.078	C/G	Intron 21	C/G in three unrelated samples, G/G in two siblings (family 3)
rs6449209	A = 0.417 G = 0.583	A/G	Intron 22	A/G in families 1 and 2 and in three unrelated subjects, A/A with +G in family 3

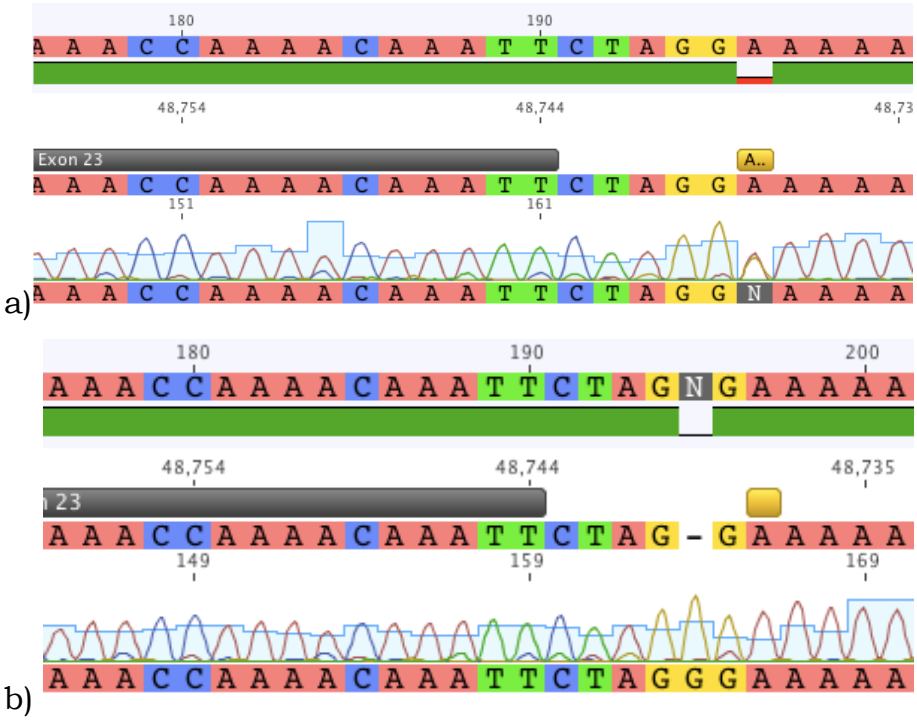


Figure 10.3 a) Nucleotide trace from one subject with the normal *PROM1* sequence included above the trace and the consensus sequence highlighting the variation on the top line. The A/G variation upstream of exon 23 is designated as rs6449209 and was seen in all subjects except one family: b) shows the nucleotide trace from one of three siblings that showed an insertion of G prior to this SNP.

***PROM1* Haplotypes**

In order to refine the haplotype of the *PROM1* genomic region, the marker D4S1601, identified as being linked to *PROM1*, was screened in affected families. The new British families were found to have the allele with 135 repeats, whilst the previous British family had a distinct allele with 129 repeats. This suggests the three new families have a common ancestral haplotype, whereas the previously identified family does not. These results have been presented in Michaelides et al. (2010).

RIMS1

Two of the MCDR2 subjects that lacked a *PROM1* mutation were screened for mutations in exon 14 of *RIMS1* (Johnson et al., 2003) but no changes were found.

10.3.2 BEM Panel

Fourteen individuals diagnosed with bull's-eye maculopathy (BEM) were screened for mutations in exon 10 of the *PROM1* gene but none were found.

10.4 Discussion

MCDR2 is an autosomal dominant form of bull's-eye maculopathy characterised by annular RPE atrophy with central sparing of the fovea. *ABCA4* sequence variants have been described as causing BEM (Michaelides et al., 2007) but MCDR2 was linked to 4p15-p16.3 (Michaelides et al., 2003b) and the mutation Arg373Cys in *PROM1* identified (Michaelides et al., 2003a). This mutation has been linked to three other forms of retinal dystrophy; RP (Michaelides et al., 2010), STGD4 and CORD (Yang et al., 2008). A panel of BEM families with suspected MCDR2 were screened for mutations in all *PROM1* coding exons. Other members of the panel

were screened only for mutations in exon 10 (the c.1,117 C>T mutation that leads to Arg373Cys). Though nucleotide variations were identified in the screening process, they generally occurred at known SNP sites (Table 10.4). The exception was a homozygous change in exon 19 not annotated in Ensembl, seen in one individual. The two siblings of this individual did not carry the allele and were homozygous for the common nucleotide. The nucleotide change would not alter the amino acid sequence so it is not thought to be associated with the disease. Despite no new mutations being found, three new families with the c.1,117C>T mutation were identified (only two families, D and E, were described in Michealides et al., 2010). All three new families with the Arg373Cys mutation had the 135 haplotype for the marker D4S1601, which is linked to *PROM1*. This marker haplotype was also seen in the RP family with BEM reported by Michaelides et al (2010) but the original British MCDR2 family had a 129 haplotype.

PROM1 is a membrane-associated protein normally found at the base of outer segment disk membranes in the photoreceptors where new disks are formed (Figure 10.4). Yang et al. (2008) generated transgenic mice with the Arg373Cys mutation in human *PROM1* under the control of the rhodopsin promoter to achieve exclusive expression in the rod photoreceptors. They found that rather than PROM1 being at the outer segments, endogenous protein gathered in the photoreceptors. Combined with evidence of overgrown and disorientated disk membranes they suggested PROM1 may be responsible for new disk formation. Yang et al. (2008) data also indicated an interaction of PROM1 with PCDH21, which suggests that mutations in this gene may also cause macular dystrophy. Mutations in *PCDH21* have been associated with autosomal recessive RP (Bolz et al., 2005).

Whilst the Arg373Cys mutation occurs in different retinal diseases, a consistent feature of the phenotypes seen is BEM. Of the two panels screened in this project, only three new families identified as MCDR2 carried this mutation, therefore there are clearly more causes of MCDR2 and BEM to be identified. Screening of *RIMS1* exon 14 (a mutation in which causes CORD, Johnson et al., 2003) revealed no variations in the MCDR2 subjects screened.

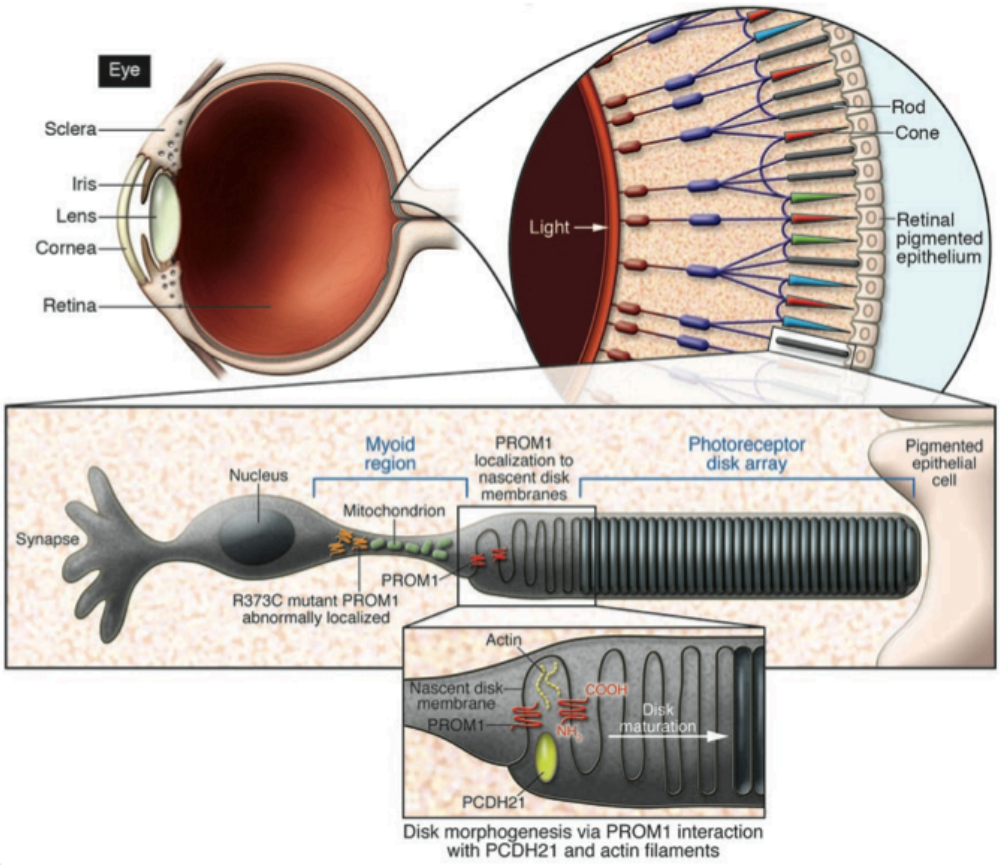


Figure 10.4 Taken from Kleinman and Ambati (2008) showing that PROM1 normally localises at nascent disk membranes but with the Arg373Cys mutation it remains in the myoid region.

In summary, of one panel of MCDR2 and one of BEM subjects, three new families with the Arg373Cys mutation in *PROM1* were

identified. No new mutations in *PROM1* were detected in the MCDR2 panel screened. It is evident that mutations in other genes yet to be identified exist that cause BEM, an interesting candidate appears to be *PCDH21*.

11. BORNHOLM EYE DISEASE

11.1 Genetic Analysis

Bornholm Eye Disease (BED) is a rare non-progressive, X-linked disorder in which patients exhibit the key features of myopia, cone dysfunction and dichromacy. Families have been identified with both protanopic and deuteranopic dichromacy with the other features of the disorder common to all. The BED locus has been linked to Xq28 (Schwartz et al., 1990 and Young et al., 2004), the region at which the L and M opsin gene array lies, variations in which lead to dichromacy.

The L and M cone photopigments are encoded by genes in a head-to-tail tandem array on the X chromosome with only the first two genes in the array being expressed (Section 1.2.4). The genes and expressed opsins are highly similar with spectral differences between the visual pigments reliant on just seven amino acid residues (116, 180, 230, 233, 277, 285 and 309, Asenjo et al., 1994; Section 1.2.4). Hybrid genes consisting of exons from both L and M genes therefore encode pigments with different spectral peaks to the normal L and M genes. The residues in exon 5 (277, 285 and 309) determine whether the pigment functions predominantly as an L or M opsin.

Mutations in either of the L or M genes, either by amino acid change or gene deletion/replacement, are associated with dichromacy (Section 1.2.4). The most frequently reported mutation in these opsin genes is the substitution of an arginine for a cysteine at position 203 (Cys203Arg), which renders the pigment non-functional (Kazmi et al., 1997). Both Young et al. (2004) and Michaelides et al. (2005) identified this mutation in

families exhibiting BED but though the phenotype was consistent across families, this mutation was not. This finding suggested that different mutations in the L or M genes could be responsible for BED in other families. Nathans et al. (1989) previously observed that the combination of amino acids leucine, isoleucine, alanine, valine, alanine (LIAVA) at 153, 171, 174, 178 and 180, respectively, may be a cause of blue cone monochromacy (BCM). BCM results when individuals express a functional S opsin only and affected members of one family reported by Nathans et al. (1989) had a single X-linked visual pigment gene with the LIAVA exon 3 combination and this co-segregated with absence of function of the corresponding cones. In 2004, Neitz et al. reported on a dichromatic individual with cone dysfunction with this combination of amino acids encoded by their M gene. The LIAVA amino acid combination in exon 3 was not observed in more than 300 L and M pigment gene sequences from individuals with normal colour vision, as reported by Neitz et al. (2004). Other publications of exon 3 amino acid combinations in dichromats also show no evidence of the LIAVA combination (Sharpe et al., 1998; Ueyama et al., 2004). It was suggested that this combination of amino acids at these polymorphic sites was inactivating the opsin, causing both the dichromacy and cone dysfunction. Carroll et al. (2004) published adaptive optics evidence on this individual that revealed there was in fact a loss of cones from the retina. In this subject the LIAVA appeared to result in a non-functional M opsin and patchy loss of cones was seen throughout the cone mosaic.

Based on these reports it was hypothesised that the BED subjects may have the combination of LIAVA encoded by either the first or second gene in their opsin arrays, leading to loss of the

corresponding cone class and resulting in both dichromacy and cone dysfunction.

11.2 Methods Applied

11.2.1 L/M Exon 3 Screening

BIOTaq PCRs were conducted to amplify exon 3 of the L and M opsin genes using the primers in Table 11.1 and a 60°C annealing temperature. Amplicons were gel-extracted and ligated into pGEM-T vectors before being transformed into competent JM109 cells, following which around 50 colonies were selected and the plasmids extracted and inserts sequenced (for cloning protocol, see Section 3.3).

11.2.2 L/M Exons 3-5 Screening

The BIOXACT Long range with HiSpec protocol was used (Section 3.1.4) to amplify across exons 3 and 5, a distance of 3,809 nucleotides. Reactions were conducted with a 62°C annealing temperature using the exon 3F and 5R primers listed in Table 11.1. The amplicons were then cloned, extracted and sequenced as in Section 11.2.1 but with 30 colonies selected.

11.2.3 Amplifying First and Downstream Genes of the L/M Array

Primers specific to the promoter region of the upstream L gene and downstream sequence following exon 6 were used to amplify the first gene in the array (Section 3.1.6, primers listed in Table 11.1, taken from the publication by Oda et al., 2003). For the amplification of the downstream genes, the forward primer was designed to be specific to the downstream promoter (Table 11.1) and both reactions used the same reverse primer. When visualised on a 0.5% gel, the bands were often feint but visible nonetheless.

These PCR reactions were then used as the DNA template for subsequent BIOTaq reactions for the amplification of exons 2, 3, 4 and 5 using the primers listed in Table 11.1 (annealing temperatures 60°C). The amplified exons were then sequenced using the BigDye protocol (Section 3.1.1).

Table 11.1 Primers used for the various amplifications of the L/M gene arrays.

Primers	Name	Use
GAGGAGGAGGTCTAAGTCCC	Promoter F	L to M ratio using *Bsr* FI
GGCTATGGAAAGCCCTGTCCC	Promoter R	
AGTCCCAGGCCCAATTAAGA	1F	Screening of exon 1
CCCAATTCATGTCATCAGA	1R	
GCACTGGTATAGACAGGCG	2F	Screening of exon 2
CAGTATATGGATGTGAGGC	2R	
CTCAGTCCGTGGAGCCCTGAATTC	3F	Screening of exon 3 and exons 3 to 5 with BIOXACT Long
ACATTGATAGACATTGCACGCTCA	3R	
GGTGACTGCCACAGAATTGAT	4F	Screening of exon 4
CTGATTCTCATCGCTGGATCT	4R	
CTATGCCTGGGTCACCTGCCTC	5F	Screening of exon 5 and exons 3 to 5 with BIOXACT Long
CTTATCAGAGACATGATTCCAGGTGG	5R	
GCACGTACATTCAGCACAG	6F	Screening of exon 6
CACGCAGAGGCTCAGGTC	6R	
GAGGCGAGGCTACGGAGT	FG	LR PCR of the first genes
GCAGTGAAAGCCTCTGTGACT	E6	
TTAGTCAGGCTGGTCGGGAACT	DG	LR PCR of the downstream genes
GCAGTGAAAGCCTCTGTGACT	E6	
TTTGCTGCTGCCAACCCT	L/M F	QPCR amplification of L and M exon 5 amplicon
TTGCTTACCTGCCGGTTCATAA	L/M R	
CCTGCCGGCCTACTTTGCCAAA	L Probe	Probe for L exon 5
CCTGCCGGCCTTCTTTGCCAAA	M Probe	Probe for M exon 5

11.2.4 Ratio of L to M Genes

The method published in Ueyama et al. (2004) was initially attempted to determine the ratio of L to M genes (Section 3.1.10), which involved amplifying the promoters of the L and M genes

using the same set of primers and the GoTaq PCR protocol (Table 11.1). These amplicons were then digested with *Bsr* F1 and the digested products run on a low-melt agarose gel and stained with SYBR Green I (Figure 11.1).

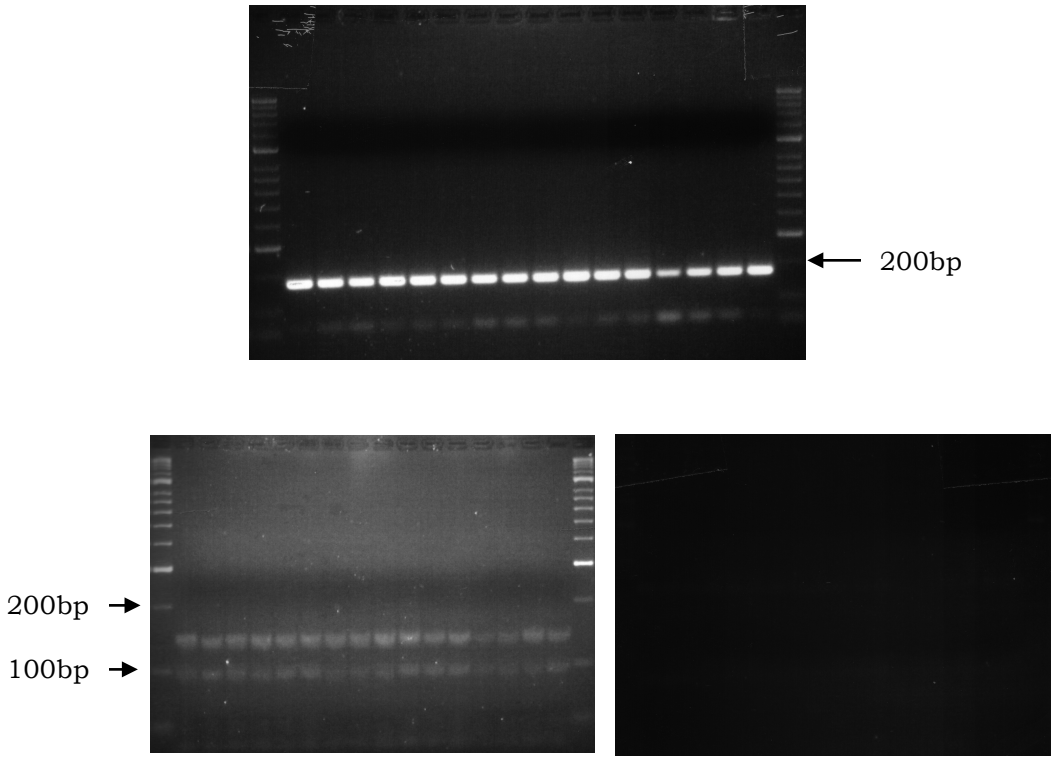


Figure 11.1 Gel images showing: top; the PCR products of the promoter amplifications and bottom; staining of *Bsr* F1 digested L and M promoters with ethidium bromide (left) and SYBR Green (right).

11.2.5 TaqMan L and M QPCRs

This method was taken from Neitz and Neitz (2000) and is outlined in Section 3.1.8. The same set of primers were used to amplify exon 5 of the L and M genes with the addition of probes specific for either the L or M exon 5 sequence (Table 11.1). The delta-delta-Ct method was used to determine the ratio of L exon 5 to M exon 5 in a sample of genomic DNA. Experiments were repeated on three separate occasions.

11.3 Results

11.3.1 Outline of Analysis

Exon 3 Screening

Initially, 12 subjects from four BED families (one of which was deuteranopic) and an unrelated BED protanope (Subject Y) were screened for variations in exon 3 of their L and M opsin genes. In order to separate the L and M sequences, the amplicons were cloned and around 50 colonies selected for screening. 50 colonies were selected to ensure that all forms of L and M sequences were identified and to determine that any variations in coding sequence seen were genuine and found in more than one colony. In all but three samples (from the same protanopic family), this screening identified a combination of nucleotide changes that would result in a specific amino acid combination not seen in normal trichromats of: LIAVA at amino acid sites 153, 171, 174, 178 and 180, respectively. Figure 11.2 shows the nucleotide trace for one of the affected males from the deuteranopic family and the amino acid changes that would be encoded. The common sequence at these amino acid sites is leucine, valine, alanine, isoleucine and serine (LVAIS). All samples had the combination methionine, valine, valine, valine, alanine (MVVVA) at these sites in their M exon 3 (the common sequence is MVAIA) but this variant is occasionally seen in the normal population.

For the protanopic family that did not show this combination of amino acids, exon 4 was screened and the mutation that results in the Cys203Arg amino acid substitution identified, as found previously in this family by Michealides et al. (2005). No other variations were found in this family.

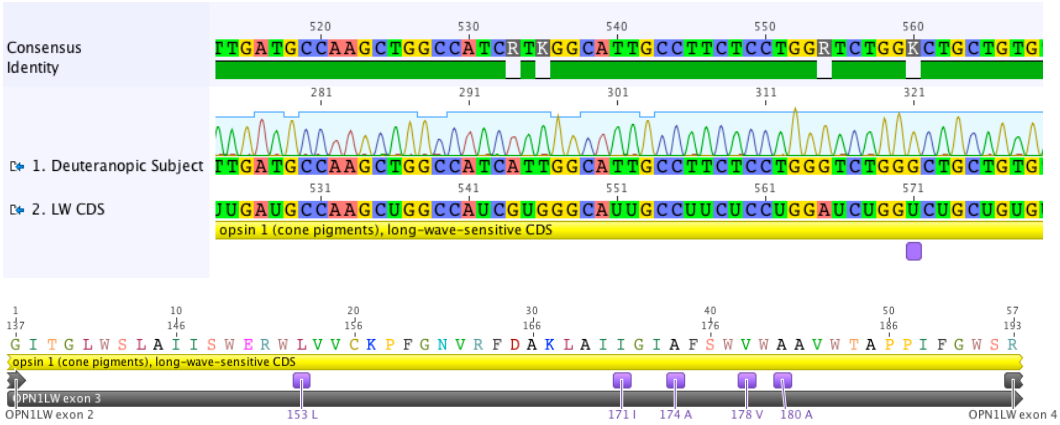


Figure 11.2 The top image shows the alignment of the nucleotide trace from a deuteranopic BED male with the L opsin exon 3 sequence. These nucleotide changes result in amino acid substitutions at sites 171, 178 and 180 shown in the bottom image (the normal sequence at the sites highlighted in purple is: LVAIS).

Exon 3 to 5 Screening

In order to determine whether the LIAVA combination encoded by exon 3 was linked to an L or M exon 5, exons 3 to 5 were amplified in a single amplicon, cloned and screened (Section 11.4.2). In order to ensure no sequences were missed, up to 30 clones were isolated and used for screening. Given the similarities between the L and M exon and intron sequences, formation of chimeric sequences from the exon 3 to 5 amplifications was possible (Figure 11.3). Such PCR artifacts would result in false hybrids. Chimeric amplicons can be formed when synthesis of a nascent strand begins on one template but becomes interrupted. If this strand shows homology to another template it can bind to this and amplification continue from this strand. The potential for this in the PCRs conducted in this section is high given the sequence homology between the L and M genes. Sequences found in a number of clones were predicted to be genuine and in general, this has been subsequently confirmed. Predictions of the ratio of L to M were also made from the cloning data.

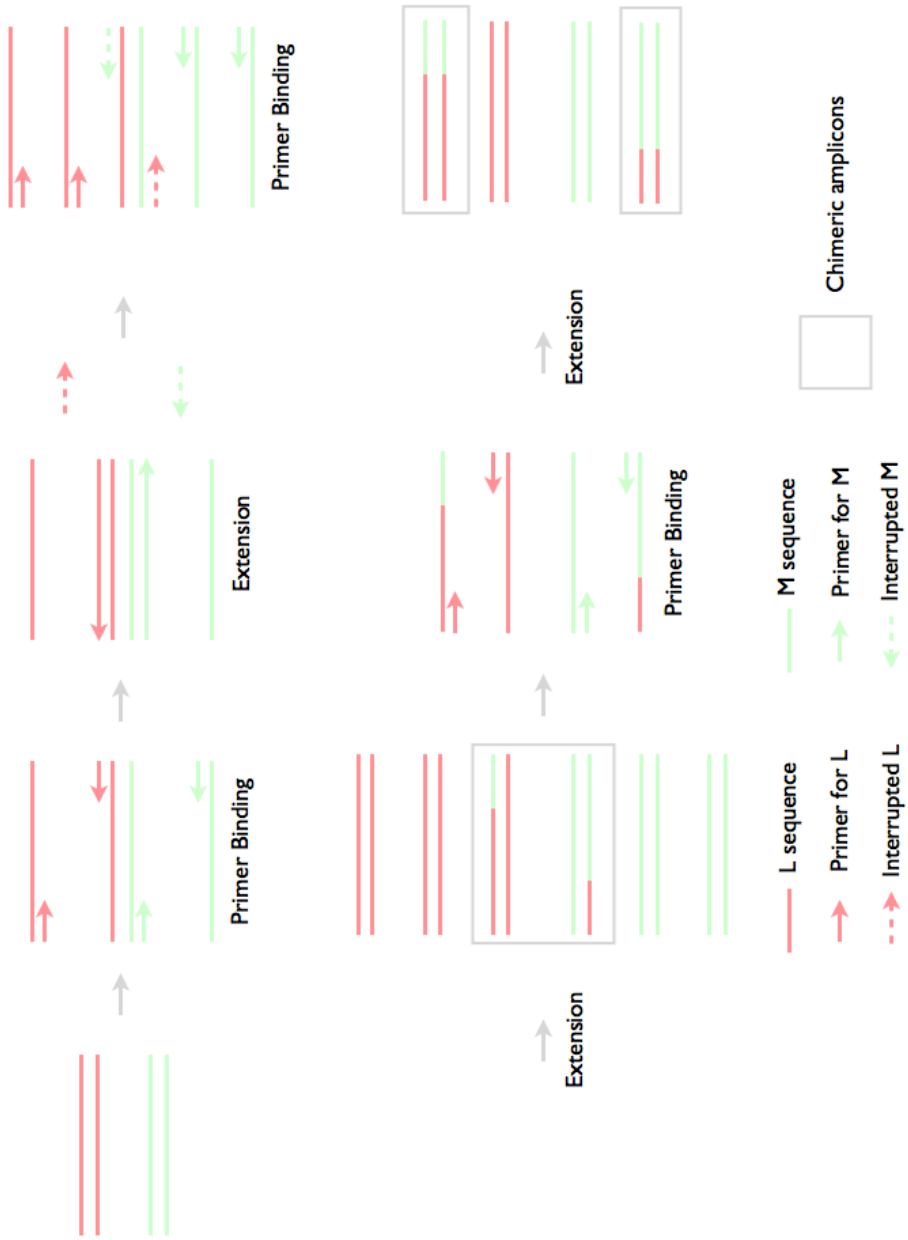


Figure 11.3 Diagram depicting the production of chimeric PCR artefacts from homolgous sequences.

Long Range PCRs

In order to validate the cloning data and accurately determine gene structure of the L and M arrays in the subjects, the method published by Oda et al. (2003) was used. The protocol for these long-range PCRs is described in Section 3.1.6. Essentially, primers specific to the promoter region of the upstream L gene and the region following exon 6 were used to amplify the first gene in the array (primers listed in Table 11.1 taken from the publication by Oda et al., 2003). The position of the primer for the first gene amplification is shown in Figure 11.5. For the amplification of the downstream genes, the forward primer was designed to be specific to the downstream promoter (Table 11.1) and used the same reverse primer as for the first gene amplification. When visualised on a 0.5% gel, the bands were often feint but visible. These PCR products were then used as the DNA template for subsequent BIOTaq reactions for the amplification of exons 2, 3, 4 and 5 (annotated in Figure 11.4).

L to M Ratio

Attempts were made to determine the ratio of L to M genes. Initially, the method published by Ueyama et al. (2004) was used. The gels were stained with ethidium bromide to ensure the digestion of the PCR products was successful (Figure 11.1). Unfortunately, the fluorescence with SYBR green failed, so the relative amounts of product could not be determined.

As this method was unsuccessful in determining the ratio of L to M genes, the TaqMan QPCR method described by Neitz and Neitz (2000) was used. This method involved amplifying the L and M exon 5 sequences in the same reaction with the same set of primers (Figure 11.5).

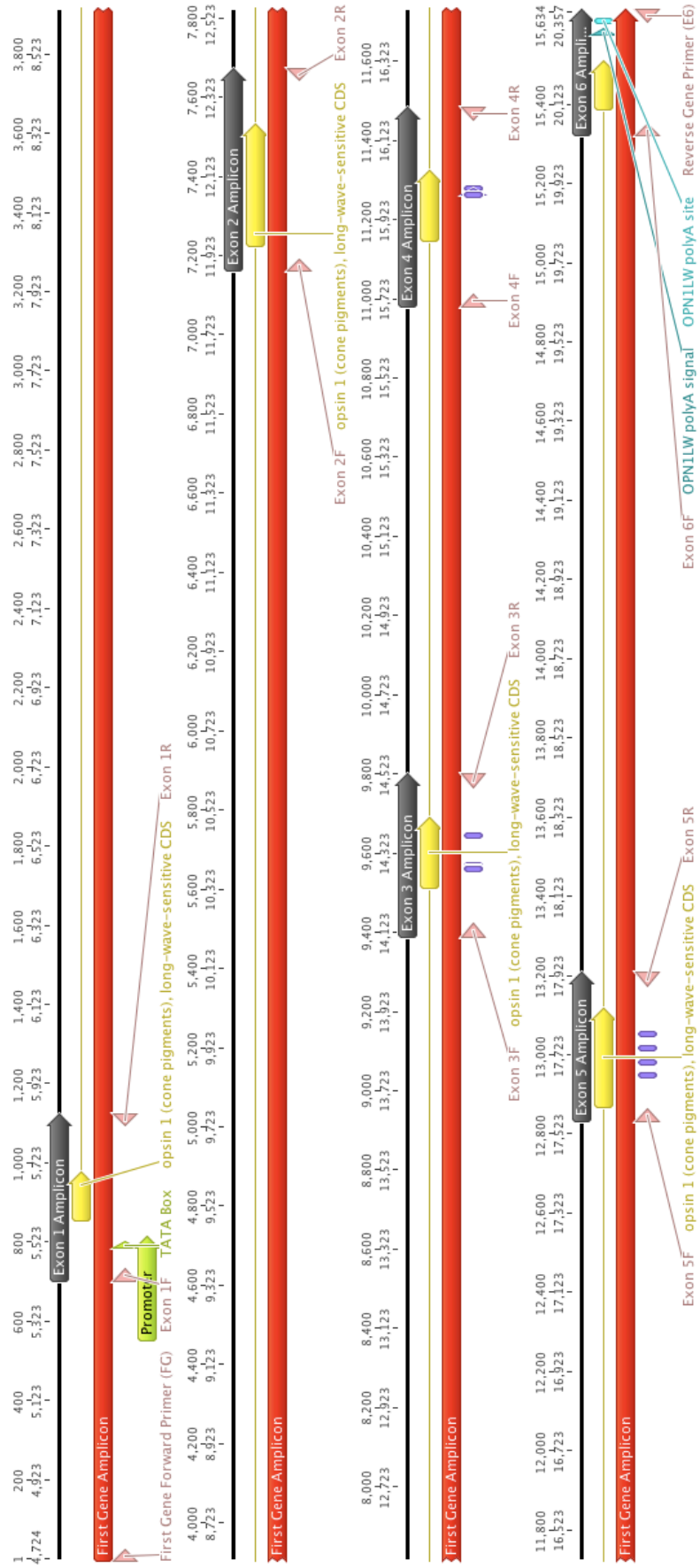


Figure 11.4 Image from Geneious (Drummond et al., 2010) showing the relative positions of L opsin gene exons and the primers listed in Table 11.1. The Grey annotations indicate the amplicons amplified from the main first gene amplification (in red). Purple annotations indicate differences between L and M opsin sequences.

Figure 11.5 Image showing the L gene exon 5 sequence with the region amplified by the TaqMan primers highlighted in grey. The probe positions are also annotated.

Each reaction included one probe (labelled with 6FAM) that would specifically bind to the L exon 5 sequence and a second probe (labelled with JOE) that would specifically bind to the M exon 5 sequence. The probes anneal to the single strands of their specific exon 5 sequences and as Taq polymerase extends the primer and creates the new strand it encounters the probe and begins to degrade it. This releases the fluorophore from the 5' end of the probe, levels of which are detected following each amplification cycle. Prior to degradation, the fluorophore is prevented from fluorescing by a quencher on the 3' end of the probe (TAMARA). The fluorescence detected is therefore directly proportional to the fluorophore released and the amount of DNA template in the PCR. The raw data obtained from these QPCRs were analysed using the delta-delta-Ct method to determine to ratio of L to M genes (Section 4.5). The L gene amplification was used as the reference gene and a sample from the Deuteranopic Family used as the "control" DNA sample. The QPCR data were combined with the results from the long range PCRs to predict the array structures, as shown in Table 11.13.

Long range PCR results were consistent in replicate experiments with only Offspring 2 of Protanopic Family 1 failing to have first gene and downstream gene sequences separately amplified and sequenced. This occurred in replicate experiments so seems unlikely to be due to experimental error. It may be that the promoter regions to which the primers for the first and downstream genes specifically bind are altered in this subject, resulting in primer binding being non-specific to the opsin arrays.

11.3.2 Deuteranopes

Family 1

Family 1 consisted of a mother and two affected male offspring. The results of the exon 3 to 5 cloning are shown in Table 11.2. All three samples produced a number of hybrid sequences, with some of these represented by only a few clones. For example, the L, L3M4, M4L5 and M sequences were found in each sample, with L, L3M4 and M found at the highest frequencies. This suggests that these were amplified directly from corresponding genes whereas the others may be hybrid artifacts. A summation of L and M exon 5 sequences across the clones gives an indication of the ratio of these L and M exons within each opsin gene array, but does not give any indication of gene order within each array. Long-range PCRs were conducted for this purpose and the results are shown in Table 11.3.

Table 11.2 Sequences extracted from clones containing amplicons of exons 3 to 5 of the L and M genes from genomic DNA of a Deuteranopic Family 1.

	Genotype	Clones	153, 171, 174, 178, 180 Amino Acid Combination	L	M	Approximate Ratio
Mother	L3M4	7	LIAVA		7	
	L4M5	3	LIAVA		3	
	M4L5	1	MVVVA	1		
	L3M4L5	2	LVAIS	2		
	M3L4M5	2	MVVVA		2	
	L	4	LVAIS and LIAVA	4		
	M	9	MVVVA		9	
	Total:	28		7	21	1:3
Offspring1	L3M4	11	LIAVA		11	
	M4L5	2	MVVVA	2		
	L3M4L5	2	LIAVA	2		
	M3L4M5	1	MVVVA		1	
	L	4	LIAVA	4		
	M	6	MVVVA		6	
	Total:	26		8	18	1:2
Offspring2	L3M4	10	LIAVA		10	
	L4M5	4	LIAVA		4	
	M4L5	1	MVVVA	1		
	L	5	LIAVA	5		
	M	8	MVVVA		8	
	Total:	28		6	22	1:4

Table 11.3 Data from the long range PCRs conducted with DNA from the Deuteranopic Family 1 are shown. The identities (L or M) of each exon screened are given and the combined genotypes of the first and downstream genes are shown. Each experiment was repeated.

	First Gene Sequence				Genotype	Downstream Gene Sequence				Genotype
	2	3	4	5		2	3	4	5	
Mother	L (LIAVA) & L (LVAIS)		L	L	L (LIAVA) and L (LVAIS)	L (LIAVA) & M (MVVVA)		M	M	L3M4 (LIAVA) & M (MVVVA)
Offspring1	L (LIAVA)		L	L	L (LIAVA)	L (LIAVA) & M (MVVVA)		M	M	L3M4 (LIAVA) & M (MVVVA)
Offspring2	L (LIAVA)		L	L	L (LIAVA)	L (LIAVA) & M (MVVVA)		M	M	L3M4 (LIAVA) & M (MVVVA)

The long-range PCRs produced fewer sequence variations, confirming the suspicion that many of the sequenced hybrids were PCR artifacts. First gene amplifications for the mother identified two L genes, one with a normal sequence and one with the LIAVA combination. This is consistent with one L gene on each X chromosome. The downstream gene amplifications revealed an

L3M4 sequence that also contained the LIAVA sequence and M sequence with the uncommon MVVVA combination. Both offspring were identified as having an L (LIAVA) gene followed by L3M4 (LIAVA) and M (MVVVA) sequences, although the order of the downstream sequences was not determined. These sequences represent 21 of 26 and 23 of 28 sequences from the exon 3 – 5 amplicons for Offspring 1 and 2 respectively, indicating that the other four hybrids are PCR artifacts. To determine the relative numbers of genes within the opsin arrays, QPCRs were conducted. The carrier mother's L to M QPCR ratio was generated as 2:4, which equates to a single L and two M genes on each chromosome. Male Offspring 1 gave a ratio of 1L:4M whilst male Offspring 2 had a ratio of around 1L:2M. The data from this deuteranopic family are combined in Figure 11.6. ANOVA comparison of the Mother's L and Offspring 2 M revealed no significant difference ($p = 0.1$), which was also found for Offspring 1 and 2 L comparisons ($p = 0.233$) and the Mother's M and Offspring 1 M ($p = 0.487$). This indicates the ratios are correct.

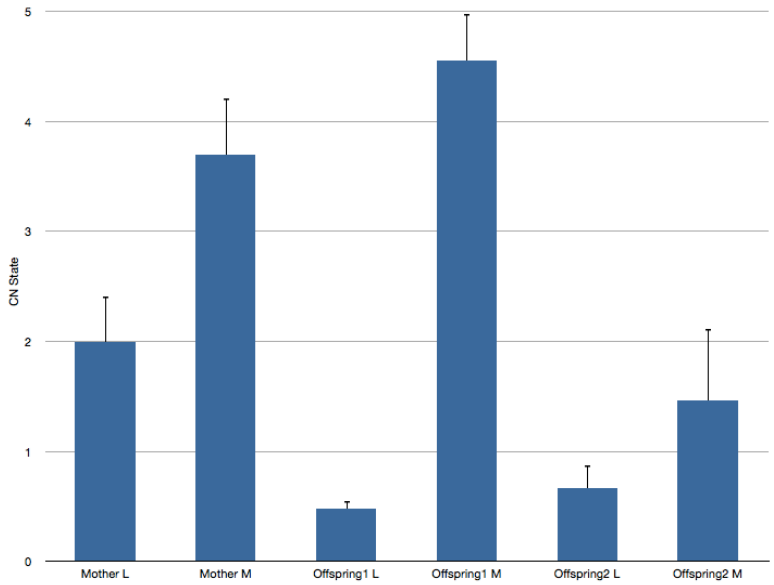


Figure 11.6 Comparisons of the exon 5 L and M ratios of the Deuteranopic Family. Error bars represent SEM.

The data for this family were consistent and revealed the mother to have one array consisting of an L (LIAVA) gene followed by L3M4 (LIAVA) and a single M (MVVVA) gene. This array was inherited by both offspring. The second of the mother's arrays is normal and contains L (LVAIS) followed by two M genes (MVVVA). In Offspring 1 it appears that a recombination occurred between the Mother's two arrays, giving the structure: L (LIAVA), L3M4 (LIAVA), M (MVVVA), M (MVVVA), M (MVVVA) and M (MVVVA). This is consistent with QPCR data that provided a ratio of 1L:4M exon 5 sequences (Figure 11.6).

The Original BED Family

A single sample from the original Danish BED family (Haim et al., 1988) was analysed. The exon 3 to 5 cloning process identified the sequences shown in Table 11.4.

Table 11.4 Sequences extracted from clones containing amplifications of exons 3 to 5 of the L and M genes from BED genomic DNA.

	Genotype	Clones	153, 171, 174, 178, 180 Amino Acid Combination	L	M	Approximate Ratio
BED	L	1	LVAVA	1		
	M3L4	5	MVVVA	5		
	M	7	MVAIA		7	
	Total:	13		6	7	1:1

L, M3L4 and M gene sequences were identified. The sequence of the L exon 3 encoded V (valine) rather than I (isoleucine) at site 171 to give the LVAVA combination. Like LIAVA, this is a rare sequence amongst both trichromats and dichromats. The L and M exon 5 frequencies in the clones gave a predicted 1:1 ratio of the L and M exon 5 sequences within the array. This ratio suggests therefore that the array contains two M genes. As the subject is

deuteranopic (lacking a functional M opsin), this leads to an array prediction of L (LVAVA), then M3L4, followed by two M sequences.

The long-range PCR data shown in Table 11.5 confirmed the cloning data and identified an upstream L gene with a nucleotide sequence that would lead to an LVAVA amino acid combination at residues 153, 171, 174, 178 and 180, respectively. The L sequence would then be followed by downstream M3L4 and M sequences. The ratio of these genes was analysed by QPCR and a ratio of 1L:1M ($p = 0.566$) exon 5 sequence detected, shown in Figure 11.7. This ratio is consistent with the long range PCR, which showed the presence of two L exon 5 sequences and two M exon 5 sequences. The QPCR results were compared to those from the Mother of Deuteranopic Family 1 (DFM1), where a ratio of two L and four M exon 5 sequences was determined. Though the difference in levels fails to reach insignificance when compared to DFM1 ($p = 0.016$), the ratio is 1:1 and combined with the long-range PCR data it appears that there are two L and two M genes in this sample. Combined, the long-range PCR and QPCR data confirm the original array structure predictions made from the cloning data. The long-range PCR data show the L (LVAVA) gene is the first gene in the array. It is suggested that this would lead to the production of a non-functional opsin and based on the deuteranopic phenotype, this means the next gene in the array would have to encode a functional L opsin. This leads to the prediction that the array structure for this subject is: L (LVAVA) followed by M3L4 then two M genes.

Table 11.5 Data from the long range PCRs for the original BED subject with the combined genotype of first and downstream genes provided. Each experiment was repeated.

	First Gene Sequence				Genotype	Downstream Gene Sequence				Genotype
	2	3	4	5		2	3	4	5	
BED		L (LVAVA)	L	L	L (LVAVA)		M (MVVVA) & M (MVAIA)	L & M	L & M	M3L4 & M

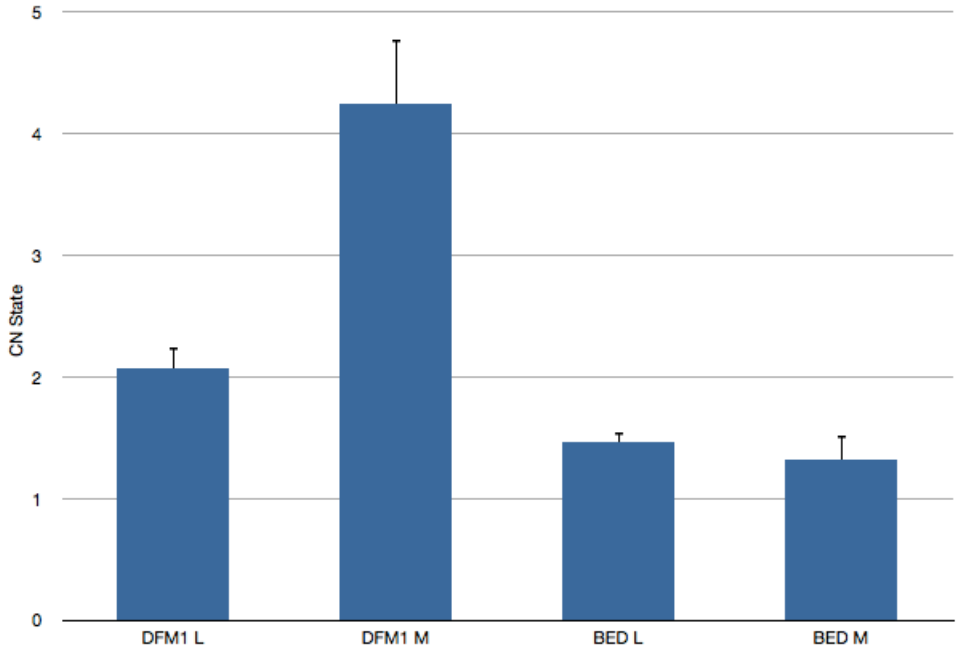


Figure 11.7 Comparisons of the exon 5 L and M ratios of the Original BED family member compared to the Deuteranopic Family Mother (DFM1). Error bars represent SEM.

Deuteranopic Subject X

A single male subject for whom data has not been previously reported was analysed. The cloning results are shown in Table 11.6 and reveal a number of different clones.

Table 11.6 Sequences extracted from clones containing amplifications of exons 3 to 5 of the L and M genes from Subject X genomic DNA.

	Genotype	Clones	153, 171, 174, 178, 180 Amino Acid Combination	L	M	Approximate Ratio
Subject X	L3M4	2	LVAVA		2	
	M3L4	3	MVAVA	3		
	M4L5	6	MVAVA	6		
	M3L4M5	2	MVAVA		2	
	L	10	LVAVA	10		
	M	7	MVAIA and MVAVA		7	
	Total:	30		19	11	2:1

As for the deuteranopic family, it was suspected that some of the sequences of the clones were PCR artifacts. As the clones of the highest frequencies in Deuteranopic Family 1 were confirmed to be the genuine clones, that would suggest the real sequences from Subject X are L (LVAVA), M4L5 and M. As neither long-range nor QPCR were conducted on this sample, the cloning data alone have been used to predict the array structure. Given the deuteranopic phenotype this subject would lack a functional M opsin, therefore a normal M gene sequence would not be in position one or two of the array. An array of L (LVAVA), M4L5, M (MVAIA) and M (MVAVA) would be consistent with the deuteranopia of Subject X. The LVAVA combination in the first L gene would be predicted to be non-functional and lead to cone dysfunction and the M4L5 hybrid gene would produce a functional L pigment. This array prediction is not entirely consistent with the 2L:1M ratio of exon 5 sequences determined by cloning. It should be noted however that the actual ratio is 1.72 L to 1 M.

11.3.3 Protanopes

Protanopic Family 1

The cloning data for this family are shown in Table 11.7. Comparison of the clones found in all three subjects suggest that the mother has an array consisting of an L gene (LIAVA) followed by at least one M (MVVVA) gene, which is inherited by both offspring. The mother would have a second array but from the cloning data it is difficult to predict its structure.

The ratios determined from the clone frequencies suggest the mother has an equal number of L and M opsin genes whilst both offspring have one L to two M opsin genes. To give an overall ratio of 1L to 1M, the mother's second array would need to be either a single L gene or two L genes with one M gene. As the LIAVA

combination is rare it would seem unlikely that the mother would have an L gene with this combination on each chromosome. As no other L gene sequence was identified this would suggest that either M3L4 or M4L5 are present in the first gene position on the mothers second array, which may or may not be followed by a second hybrid and M sequence, if the ratio determined from the clones is correct.

As with other subjects, it seemed that the cloning data and predicted ratio from this family should be treated with caution and further analysis conducted to obtain more reliable data for predicting the array structures. The long-range PCR results, summarised in Table 11.8, show that the predictions made from the cloning data appear to be largely correct. Although amplifications of Offspring 2 did not separate the L and M sequences on two separate occasions, data from Offspring 1 combined with the previous cloning data suggest that the array consists of an L (LIAVA) followed by at least one downstream M (MVVVA) gene. The mother appears to have one array of this same structure with a second consisting of M3L4 (MVVIS) followed by at least one M (MVVVA) gene.

Table 11.7 Sequences extracted from clones containing amplifications of exons 3 to 5 of the L and M genes from Protanopic Family I genomic DNA.

	Genotype	Clones	153, 171, 174, 178 and 180 Amino Acid Combination	L	M	Approximate Ratio
Mother	M3L4	3	MVVVA and MVVIS	3		
	M4L5	3	MVVVA	3		
	L3M4L5	1	LIAVA	1		
	M3L4M5	3	MVVVA and MVVIS		3	
	L	4	LIAVA	4		
	M	11	MVVVA		11	
	Total:	25		11	14	1:1
Offspring1	L3M4	1	LIAVA		1	
	M4L5	3	MVVVA	3		
	L	5	LIAVA	5		
	M	16	MVVVA		16	
	Total:	25		8	17	1:2
Offspring2	M3L4	1	MVVVA	1		
	L	5	LIAVA	5		
	M	14	MVVVA		14	
	Total:	20		6	14	1:2

Table 11.8 Data from the long range PCRs conducted with DNA from the Protanopic Family I. Identities of the sequences for each exon screened are given and the combined genotype of first and downstream genes provided. Each experiment was repeated.

	First Gene Sequence				Genotype	Downstream Gene Sequence				Genotype
	2	3	4	5		2	3	4	5	
Mother	L (LIAVA) & M (MVVIS)		L	L	L (LIAVA) & M3L4 (MVVIS)		M (MVVVA)	M	M	M (MVVVA)
Offspring1	L (LIAVA)		L	L	L (LIAVA)		M (MVVVA)	M	M	M (MVVVA)
Offspring2	L (LIAVA) & M (MVVVA)		L & M	L & M	L (LIAVA) & M (MVVVA)		L (LIAVA) & M (MVVVA)	L & M	L & M	L (LIAVA) & M (MVVVA)

QPCR data for the mother of this family consistently gave a ratio of 2:4 (Figure 11.8), which would mean the presence of two L exon 5 and four exon 5 M sequences. Analysis of the offspring data gave an L to M exon 5 ratio of 1:2 for both. ANOVA comparison of the mother's L and both offspring M samples revealed no significant difference in the copy number ($p = 0.718$), indicating the same levels of each were present. Similarly, no significant differences were seen between Offspring 1 and 2 L samples ($p = 0.268$) and M samples ($p = 0.614$).

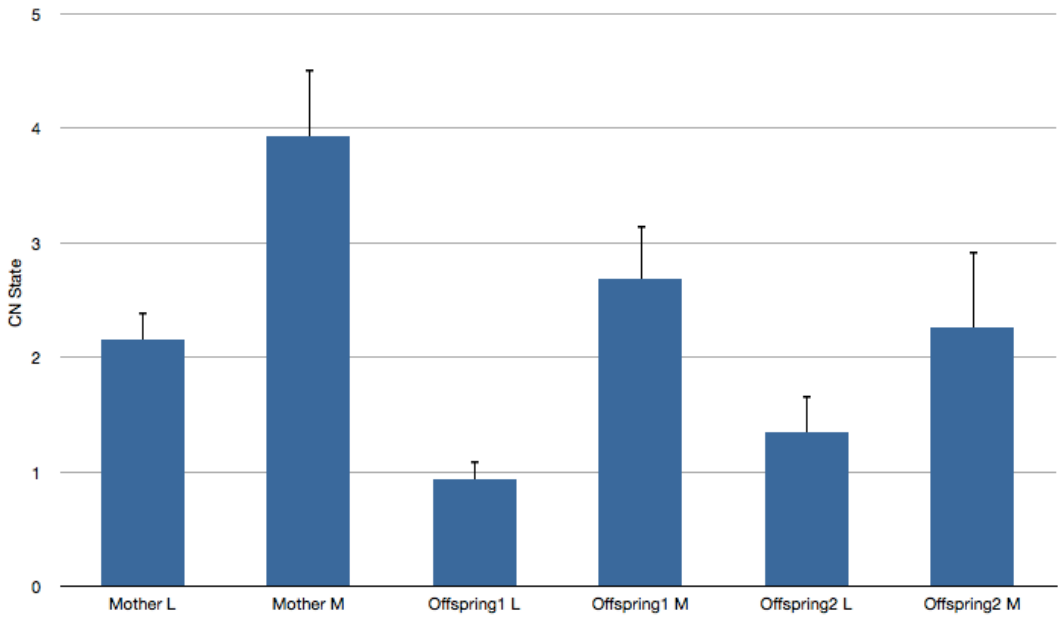


Figure 11.8 Comparison of the exon 5 L and M ratios of Protanopic Family 1. Error bars represent SEM.

Combined, it would appear that the mother has one array consisting of M3L4 (MVVIS) followed by two M (MVVVA) genes. The hybrid pigment from the first gene would function as an L opsin. The second array has an L (LIAVA) gene followed by two M (MVVVA) genes and it is this array that is inherited by both offspring. This array would also appear to encode an L and M pigment but the LIAVA combination in the L opsin would render it non-functional, leaving only a functional M pigment and giving a protanopic phenotype.

Protanopic Family 2

Long-range PCRs and QPCRs were not carried out on this family. Data from cloning of exons 3 to 5 from the two siblings are shown in Table 11.9.

Table 11.9 Sequences extracted from clones containing amplifications of exons 3 to 5 of the L and M genes from Protanopic Family 2 genomic DNA.

	Genotype	Clones	153, 171, 174, 178 and 180 Amino Acid Combination	L	M	Approximate Ratio
Sibling1	L4M5	10	LIAVA		10	
	M3L4M5	4	MVVVA		4	
	M	10	MVVVA		10	
	Total:	24		0	24	
Sibling2	L4M5	9	LIAVA		9	
	M3L4M5	4	MVVVA		4	
	M	3	MVVVA		3	
	Total:	16		0	16	

The results indicate that the siblings have a hybrid L4M5 upstream sequence with the LIAVA combination in exon 3 and two downstream M genes with the exon 3 MVVVA combination in both siblings. The data also show the presence of M3L4M5 hybrid clones in both, though the low numbers indicate that they are most likely PCR artifacts. With or without these hybrids, the structure of the array would explain the protanopia in these

males. The LIAVA combination in the hybrid L4M5 is predicted to lead to production of a non-functional protein and cause the cone dysfunction in this family.

Minnesota Family

One sample from the Minnesota Family reported on by Young et al. (2004) was analysed. The cloning of exons 3 to 5 identified an M gene and three types of hybrids (all encoding an M pigment, Table 11.10). The M sequence would appear to be genuine and it would be expected that just one of the other hybrids would also be a genuine sequence as an array consisting of more than one switch between L and M is uncommon. Given the equal frequency of the single switch clones, the array structure could not be predicted from the cloning data but seems likely that either the L3M4 or L4M5 is followed by at least one M gene sequence.

Table 11.10 Sequences extracted from clones containing amplifications of exons 3 to 5 of the L and M genes from the Minnesota subject genomic DNA.

	Genotype	Clones	153, 171, 174, 178 and 180 Amino Acid Combination	L	M	Approximate Ratio
MN	L3M4	3	LVAVA		3	
	L4M5	3	LVAVA		3	
	M3L4M5	1	MVVVA		1	
	M	13	MVVVA		13	
	Total:	20		0	20	

The long-range PCR screening results for this subject are shown in Table 11.11 and identified an upstream L gene sequence with the exon 3 combination LVAVA. This sequence was not found amongst the sequenced clones but was consistently present in the upstream long-range PCRs. The downstream sequences also contrast with the cloning data by revealing M and M3L4 genes (with two different M exon 3 sequences, one MVVVA and one

MVAIA). As with Protanopic Family 1, the presence of an L gene in the array would not normally be expected in protanopic individuals but as it contains the exon 3 LVAVA combination, it would be predicted to be a non-functional opsin. An intact M gene would therefore be expected to be directly downstream of the L gene in this array since, if M3L4 was in this second position and therefore expressed, a functional L pigment would be produced and the phenotype would not be protanopia.

Table 11.11 Data from the long range PCRs conducted with DNA from the Minnesota subject. Identities of the sequences for each exon screened are given and the combined genotype of first and downstream genes provided. Each experiment was repeated.

	First Gene Sequence				Genotype	Downstream Gene Sequence				Genotype
	2	3	4	5		2	3	4	5	
MN		L (LVAVA)	L	L	L (LVAVA)		M (MVVVA) & M (MVAIA)	L & M	L & M	M3L4 & M

QPCR analysis determined a ratio of 1:1 L and M exon 5 sequences ($p = 0.779$, Figure 11.9). Comparison with data from the Mother of the Deuteranopic Family 1 (labelled DFM1), who has two L exon 5 and four M exon 5 sequences, suggests there are two L and two M exon 5 sequences in the array as there is no significant difference between the MN L, M and DFM1 L samples ($p = 0.198$). This ratio of 2L:2M exon 5 sequences is consistent with the long-range PCR data.

Overall, the data for this subject suggest his array consists of an L (LVAVA) gene followed by an M, M3L4 and another M gene.

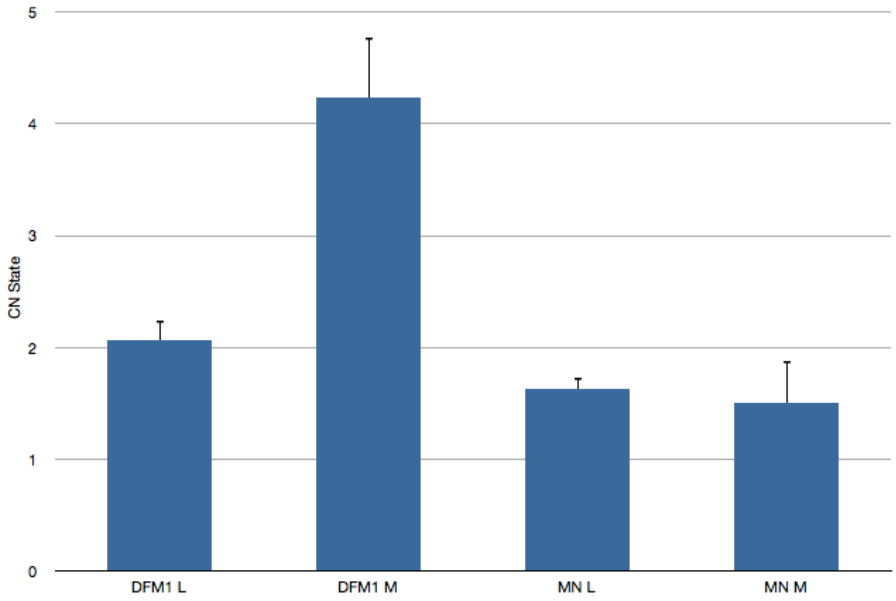


Figure 11.9 Comparison of the MN exon 5 L and M ratio compared to DFM1. Error bars represent SEM.

Protanopic Family 3

This protanopic family has not previously been investigated and screening analysis did not reveal the LIAVA or LVAVA combinations encoded by exon 3 seen in the other families. Screening for the Cys203Arg substitution revealed no mutation but screening of exon 2 revealed a new mutation of c.121G>A that would result in a Glu41Lys (glutamate to lysine) substitution. This mutation was present in both the carrier mother and affected male offspring (Figure 11.10).

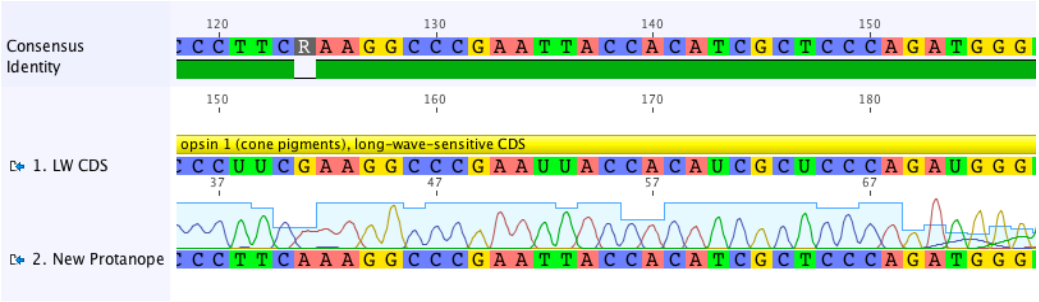


Figure 11.10 Image showing the nucleotide trace of the affected male from Protanopic Family 3 with a novel mutation in exon 2 that results in the amino acid substitution Glu41Lys.

The DNA from this family were used in long-range PCRs and the sequences identified are shown in Table 11.12.

Table 11.12 Data from the long range PCRs conducted with DNA from the Protanopic Family 3 subject. Identities of the sequences for each exon screened are given and the combined genotype of first and downstream genes provided. Each experiment was repeated.

	First Gene Sequence				Genotype	Downstream Gene Sequence				Genotype
	2	3	4	5		2	3	4	5	
Mother	L	L (LVAIS) & M (MVAIA)	L	L & M	L (LVAIS & L2M3L4M5 (MVAIA)	M	M (MVAIA)	M	M	M (MVAIA)
Offspring	L	M (MVAIA)	L	M	L2M3L4M5 (MVAIA)					

From Table 11.12 it appears that the male offspring has only a single gene in his opsin array and that it is a complex hybrid: L2M3L4M5, which would encode an M opsin and would be consistent with protanopia. The first gene screening of the mothers array also revealed this hybrid with an additional normal L gene sequence followed by normal downstream M sequences.

QPCR analysis of the L to M ratio of the Mother only successfully amplified an L exon 5 in one of the three experiments in which this was attempted, despite the long range PCRs consistently detecting an L gene sequence. This may be due to poor DNA quality and inefficient binding of the primers. The M opsin amplifications were successful and these were combined with the long range PCR data to determine the array structures for the Mother. The Offspring had no L sequence amplified, which was expected from the screening results (Table 11.12). Comparison of the mother's M exon 5 sequences to the DFM1 from revealed no significant difference ($p = 0.109$) but the QPCR data shown in Figure 11.11 suggests the mother has three copies of M exon 5 compared to one in the Offspring.

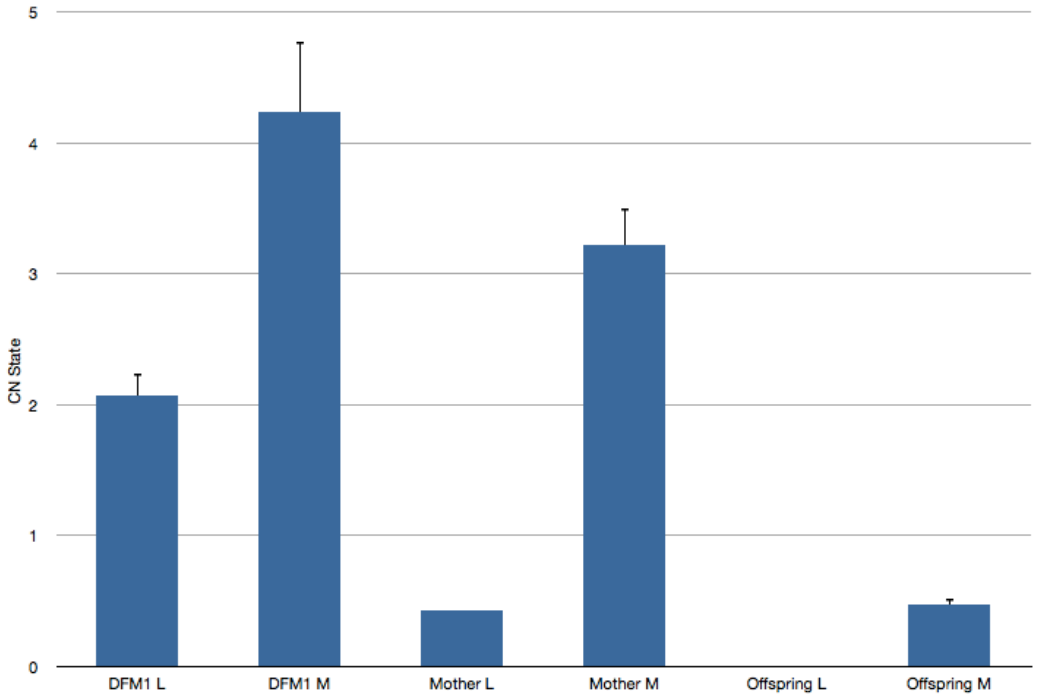


Figure 11.11 Comparison of the Mother and Offspring from Protanopic Family 3 to DFM1. Error bars represent SEM.

Combined, the long-range and QPCR data show that the male offspring of this family has a single gene in his opsin array. This gene is an L2M3L4M5 hybrid that contains a novel mutation in exon 2 that would result in a Glu41Lys amino acid substitution. To account for the cone dysfunction, the loss of functional cones would be predicted to be progressive as only a single opsin gene is present in the array and the subject does not present as a blue cone monochromat. The pigment would be expressed as a functional M, explaining the protanopic phenotype with progressive loss of cones expressing the mutated pigment causing the cone dystrophy. The mother has this array plus a second array containing a normal L gene followed by two normal M genes (based on the QPCR data).

All data are summarised in Table 11.13.

Table 11.13 Combined results of the long-range PCR and TaqMan experiments.

FAMILY		Genotype	POLYMORPHIC SITES																TAQMAN		PREDICTED ARRAY STRUCTURE
			Exon 2	Exon 3					Exon 4			Exon 5							L	M	
			41	153	171	174	178	180	230	233	236	274	275	277	279	285	298	309			
Normal L		L	E	L	V	A	I	S	I	A	M	I	F	Y	V	T	A	Y			
Normal M		M	E	M	V	A	I	A	T	S	V	V	L	F	F	A	P	F			
Deuteranopes																					
FAMILY 1	Mother	L	E	L	I	A	V	A	I	A	V	I	F	Y	V	T	A	Y			
		L3M4	E	L	I	A	V	A	T	S	V	V	L	F	F	A	P	F			L - L3M4 - M
		M	E	M	V	V	V	A	T	S	V	V	L	F	F	A	P	F	2	4	
		L	E	L	V	A	I	S	I	A	M	I	F	Y	V	T	A	Y			
		M	E	M	V	V	V	A	T	S	V	V	L	F	F	A	P	F			L - M - M
	Offspring1	L	E	L	I	A	V	A	I	A	V	I	F	Y	V	T	A	Y			
		L3M4	E	L	I	A	V	A	T	S	V	V	L	F	F	A	P	F	1	4	L - L3M4 - M - M - M
		M	E	M	V	V	V	A	T	S	V	V	L	F	F	A	P	F			
	Offspring2	L	E	L	I	A	V	A	I	A	V	I	F	Y	V	T	A	Y			
		L3M4	E	L	I	A	V	A	T	S	V	V	L	F	F	A	P	F	1	2	L - L3M4 - M
		M	E	M	V	V	V	A	T	S	V	V	L	F	F	A	P	F			
Original BED		L	E	L	V	A	V	A	I	A	V	I	F	Y	V	T	A	Y			
		M3L4	E	M	V	V	V	A	I	A	M	I	F	Y	V	T	A	Y	2	2	L - M3L4 - M - M
		M	E	M	V	A	I	A	T	S	V	V	L	F	F	A	P	F			
Subject X		L	E	L	V	A	V	A	I	A	M	I	F	Y	V	T	A	Y			
		M4L5	E	M	V	A	I	A	T	S	V	I	F	Y	V	T	A	Y			L - M4L5 - M - M
		M	E	M	V	A	I	A	T	S	V	V	L	F	F	A	P	F			
		M	E	M	V	V	V	A	T	S	V	V	L	F	F	A	P	F			
Protanopes																					
FAMILY 1	Mother	L	E	L	I	A	V	A	I	A	M	I	F	Y	V	T	A	Y			L - M - M
		M	E	M	V	V	V	A	T	S	V	V	L	F	F	A	P	F	2	4	
		M3L4	E	M	V	V	V	A	I	A	M	I	F	Y	V	T	A	Y			M3L4 - M - M
		M	E	M	V	V	V	A	T	S	V	V	L	F	F	A	P	F			
	Offspring1	L	E	L	I	A	V	A	I	A	M	I	F	Y	V	T	A	Y			
		M	E	M	V	V	V	A	T	S	V	V	L	F	F	A	P	F	1	2	L - M - M
	Offspring2	L	E	L	I	A	V	A	I	A	M	I	F	Y	V	T	A	Y			
		M	E	M	V	V	V	A	T	S	V	V	L	F	F	A	P	F	1	2	L - M - M
FAMILY 2	Sibling1	L4M5	E	L	I	A	V	A	I	A	M	V	L	F	F	A	P	F			
		M	E	M	V	V	V	A	T	S	V	V	L	F	F	A	P	F			L4M5 - M(n)
	Sibling2	L4M5	E	L	I	A	V	A	I	A	M	V	L	F	F	A	P	F			
		M	E	M	V	V	V	A	T	S	V	V	L	F	F	A	P	F			L4M5 - M(n)
MN		L	E	L	V	A	V	A	I	A	V	I	F	Y	V	T	A	Y			
		M3L4	E	M	V	A	I	A	I	A	M	I	F	Y	V	T	A	Y	2	2	L - M - M3L4 - M
		M	E	M	V	V	V	A	T	S	V	V	L	F	F	A	P	F			
FAMILY 3	Mother	L2M3L4M5	K	M	V	A	I	A	I	A	M	V	L	F	F	A	P	F			L2M3L4M5
		L	E	L	V	A	I	S	I	A	M	I	F	Y	V	T	A	Y	1	3	
		M	E	M	V	A	I	A	T	S	V	V	L	F	F	A	P	F			L - M - M
	Offspring	L2M3L4M5	K	M	V	A	I	A	I	A	M	V	L	F	F	A	P	F	0	1	L2M3L4M5
Subject Y		L3		L	I	A	V	A													

11.4 Discussion

11.4.1 L/M Mutations and Array Structure

One deuteranopic and four protanopic families with BED were analysed in addition to unrelated deuteranopic and protanopic subjects, including one sample from the original Danish BED and Minnesota families reported on by Young et al. (2004). All subjects analysed were diagnosed with the BED phenotype and found to have either a point mutation or combination of polymorphisms in one of the first two genes in their opsin array. A Cys203Arg mutation was identified in a protanopic family that was previously reported by Michaelides et al. (2005) and so not included in this analysis. The Cys203Arg mutation disrupts protein folding and stability (Kazmi et al., 1997) and has been identified as causing dichromacy (Winderickx et al., 1992c) and BCM (Reyniers et al., 1995). The cysteine residue at position 203 forms a disulphide bridge with the cysteine residue at 126 (Figure 11.14), which the substitution of arginine at 203 disrupts (Kazmi et al., 1997).

The rare L exon 3 combinations of LIAVA and LVAVA at sites 153, 171, 174, 178 and 180, respectively, were identified in all but one family with a BED phenotype and are highlighted in Figure 11.12. It can be seen that position 153 is on the cytoplasmic side of the membrane at the end of helix 3, whilst sites 171, 174, 178 and 180 are within helix 4. Residues highlighted in black represent sites at which amino acids differ between L and M opsins. From the positions of the amino acids that make up the LVAVA and LIAVA combinations seen in the BED families, it may be that these combinations disrupt the transmembrane structure, producing a non-functional opsin (causing dichromacy) which subsequently leads to degeneration of the cone photoreceptor resulting in cone dysfunction (Carroll et al., 2004). The LVAVA

and LIAVA combinations were consistently found in BED families in the upstream L gene. In one deuteranopic family, an additional LIAVA sequence was found downstream in an L3M4 gene, which was assumed to be second in the array based on the phenotype. However, as the L3M4 hybrid also contains the LIAVA exon 3 combination, it would be expected that this hybrid opsin would also lead to expression of a non-functional opsin. If both genes are non-functional then the males of this family would be expected to exhibit blue cone monochromocy, but this is not the case. Whilst they show apparent deuteranopia, they also revealed protanomolous matches made with anomaloscopy, a finding that has been previously reported in deuteranopes (Smith et al., 1979). It may be that the LIAVA combination in a hybrid L/M opsin has more of an effect than in a complete L opsin, leading to these patients being deuteranopic with protanomolous matches as opposed to being protanopic with deuteranomolous matches.

Another interesting finding is that all the BED families exhibited the combination MVVVA at sites 153, 171, 174, 178 and 180, respectively, in their downstream M opsin exon 3 sequence. Though this combination has been reported in dichromats (Sharpe et al., 1998; Ueyama et al., 2004), it is an uncommon combination and therefore finding it in all BED families is unexpected. The significance of this is unknown but it is interesting that sites not commonly polymorphic in dichromats exhibit changes in both L and M exon 3 sequences in BED families. These exon 3 M amino acids were identified as being in linkage disequilibrium with the mutation Trp177Arg in an X-linked CORD family (Gardner et al., 2010).

One family analysed (Protanopic Family 3) showed neither the Cys203Arg missense mutation nor the LVAVA/LIAVA combination

of polymorphisms in exon 3. The mother has a normal array of an L (LVAIS) and two downstream M (MVAIA) genes. Her second array comprises a single complex hybrid of L2M3L4M5, which was inherited by her male offspring. This would generate an M pigment as amino acids encoded by exon 5 are largely responsible for the peak sensitivity, though the presence of L4 might shift the wavelength absorption of the opsin. Having just this single gene in his X chromosome array would explain the protanopia but not the cone dysfunction aspect of the phenotype. Unlike the other families analysed, there were no polymorphisms in exon 3 and neither was the Cys203Arg substitution present in exon 4, instead a novel mutation in exon 2 was identified. This is not thought to completely disrupt the opsin function as does the Cys203Arg substitution because if this were the case it would be expected that the subject would present as a blue cone monochromat but he does not. It is therefore suggested that the mutation (Glu41Lys) causes a progressive loss of the pigment and, as a result, the cones expressing it. Interestingly, the cone mosaic structure of this subject was revealed by adaptive optics to present more like that of a BCM case than a BED (personal communication, Joseph Carroll). This is consistent with the idea that the hybrid L/M opsin is expressed but then progressively causes cone loss. The residue at site 41 that is changed from the exon 2 nucleotide change is shown in Figure 11.12 and lies on the extracellular side of the membrane. In the rhodopsin molecule this site is equivalent to site 25, which is close to the well known RP mutation caused by a Pro23His substitution (Figure 11.13). It is therefore unsurprising that such a mutation in the cone opsin gene would result in disruption of the opsin and lead to cone dysfunction.

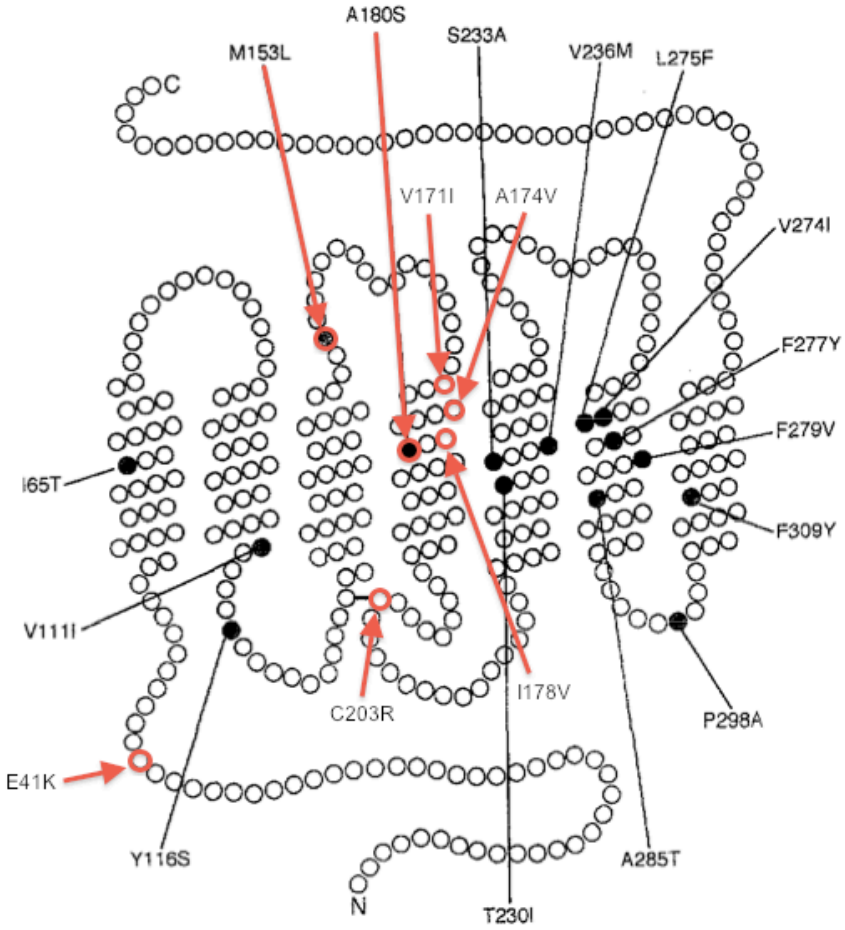


Figure 11.12 Schematic diagram taken from Asenjo et al. (1994) showing the structure of the human red and green colour vision pigments. Highlighted in red are the mutations and variations identified in the Bornholm families.

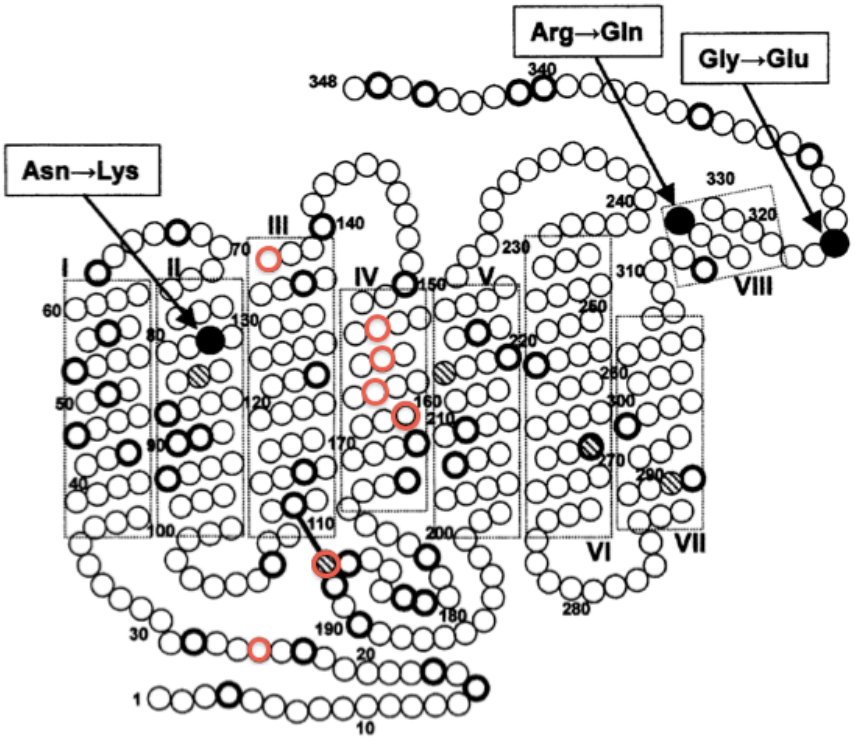


Figure 11.13 Schematic taken from Ueyama et al. (2002) showing the secondary structure of rhodopsin. Rhodopsin mutations are highlighted by a bold black outline or a full black circle. Mutations in cone opsins are shaded and the mutations from this study highlighted in red. The novel Glu41Lys mutation lies near the common Pro23His rhodopsin mutation.

11.4.2 Summary

All the subjects analysed had a BED phenotype and all were revealed to have a single sequence variation in exon 2 or 4 or a combination of variations in exon 3 of either an L or M gene in their X chromosome opsin array. Cys203Arg and LIAVA have been previously reported to cause cone dysfunction but here the combination LVAVA and Glu41Lys have also been identified. This project hypothesised that the opsin gene in which sequence variations are present leads to production of a non-functional opsin (leading to dichromacy) and causes loss of the cone in which it is expressed (cone dysfunction). The data presented suggest the hypothesis is correct and that the LIAVA combination of amino

acids in exon 3 of either an L or hybrid L/M gene cause BED. In addition to this, the variants LVAVA and Glu41Lys also appear to lead to BED.

12. DISCUSSION

Overall Summary and Discussion

This project has attempted to gain insight into the genetic causes of a number of macular dystrophies and a cone dysfunction disorder. This type of analysis is conducted in order to gain a better understanding of the retina and disease. Discovering genetic causes opens the door to investigating new pathways and functions in the retina. By identifying disease-causing genetic faults the pathology of disorders can also be better understood. A better understanding of disease pathology leads to the identification of targets for treatment and therapies. Being able to offer treatment or therapy for previously untreatable blinding disorders can dramatically improve the life of the patients. For the disorders investigated here, identifying the genetic cause has been somewhat restricted by the number of investigations that were attempted. There have been additional issues raised by the manner of the analysis conducted but this has not prevented the analysis being useful in progressing the identification of the genetic causes of the diseases.

This project has largely focused on the use of Affymetrix SNP chip technology in refining disease loci in autosomal dominant families suffering from a macular disorder. For the disorder North Carolina macular dystrophy, scientists have been trying for years to uncover the genetic basis of the disease. A new approach was attempted here in which affected members from ten unrelated families had their genotypes at the disease locus compared in an attempt to refine the disease region. This was an incorrect approach and failed to provide informative comparisons. This analysis would have benefited greatly had unaffected samples or

parents of each proband also been analysed. This would have provided a detailed disease haplotype for each family that could then be compared. However, given the limited resources of the project, it was initially deemed worthwhile to run more affected samples from a greater number of families rather than parent-offspring pairs from half the number of families. This was an error and lack of parent-offspring data resulted in genotyping data that could not be used to aid in refining the disease locus. At SNPs where homozygous calls were given, information from the parent would be unnecessary and comparisons of genotypes between all families was conducted to provide an indication of regions of allele similarities and differences. However, without knowing the phase of alleles for SNPs with AB calls, the allele associated with the disease haplotype remains unknown. Comparison of 10 unrelated HapMap samples showed that the identification of similarities and differences between such samples can be achieved from any ten samples, highlighting how important determining haplotype is for SNP comparisons.

CNV analysis with the MCDR1 families was problematic as the samples were analysed on 50K SNP chips, which are not as reliable as 250K and SNP 6.0 arrays due to the frequency of the SNPs. The 50K chips had initially been chosen instead of the 250K chips because previous 250K analysis had shown interference between SNPs in the genotyping analysis that resulted in numerous NoCalls, which reduced the number of informative SNPs. However, subsequent MCDR3 comparisons revealed that even though the 50K data provided very few NoCalls, even with the numerous NoCalls the 250K data still provided a greater number of informative SNPs across the genome. In hindsight it would therefore seem that use of the 50K was unnecessary and that the MCDR1 samples should have been run

across the 250K arrays, which would also have allowed for reliable CNV analysis. Despite this, the CNV analysis was conducted from the MCDR1 50K data and though there are problems with the data, seven of the ten families revealed a CN state of three within the MCDR1 locus across *POU3F2* and *FBXL4*. This should not be ignored and ideally would be further investigated by other methods for all samples using SNP 6.0 or QPCR.

For the MCDR3, MCDR4 and PBCRA families studied, the Affymetrix analysis method used appears to have been more useful in refining the disease regions. By determining the inherited disease allele from parent-offspring pairs and then comparing these alleles between distant family members, exclusions of regions within the disease loci could be made. For MCDR3, this refined the region and in doing so reduced the candidate gene possibilities. Extensive bioinformatic analysis of the candidates within the disease region was conducted and primary candidates identified (*CCT5, SEMA5A, BASP1, TRIO* and *FBXL7*). The CNV analysis conducted using the 250K chip data from this family was reliable and valid though no CNVs of interest were identified. This was also the case in the MCDR4 and PBCRA analysis.

Similar analysis was conducted for the MCDR4 family with the genotyping analysis refining the large 14q11.2 locus to a region of around 300kb. Extensive bioinformatic analysis of the genes within this region was conducted and primary candidates identified (*TTC5, TEP1, CCN11P1* and *TMEM55B*). Due to the number of diseases investigated in this project, time for screening candidate genes was limited. In hindsight the option of reducing the number of diseases investigated in order to focus on finding

the genetic cause of one or two disorders in particular should have been considered.

The genetic analysis of PBCRA was very similar to that of MCDR3 and MCDR4. Comparison of the genotypes refined the locus, deduced that the disease region does directly overlap the MCDR1 locus. Despite this overlap the data suggested that *GRIK2* is an interesting candidate for PBCRA.

For these macular disorders, limited screening of candidates was conducted due to time limitations. Ideally all the primary candidates identified would have been screened but often a considerable amount of time is required to optimise PCR conditions for all primer sets and samples. The optimisation steps and obtaining the correct amplicon from the reaction can be problematic as highlighted in this project by the failure to amplify *POU3F2* and exons of other genes. General PCR conditions were first attempted (e.g. gradient BIOTaq PCRs) followed by new primer designs, reagents and PCR cycles yet all failed to produce the desired amplicon. The time undertaken to screen difficult sequences can be significant and with so many disorders and candidates within this project, not all were able to be screened. Future attempts at the failed screenings could extend to touch-down and reverse touch-down PCR methods as well as digestion of DNA with new primers designed specific to the digestion sites. It may also be worthwhile isolating mRNA from non-ocular sources for screening purposes, though this would not allow for identification of splice site or UTR mutations. A further option would be to conduct Next Generation Sequencing over the entire disease loci, which would allow screening of all candidates in one procedure.

In the SHFM and NCMD investigations, both families were small with no linkage to a particular locus, which makes the genotyping data from the 250K chips of little use other than for checks of Mendelian inheritance. These combined with CNV and QPCR analyses revealed a region of deletion followed by a duplication in the French family at 5p15.33, which lies within the MCDR3 locus. The deletion was found consistently in affected members but not in the unaffected members of the family, suggesting it may be influential in the disease state. No characterised genes lie within the area of deletion but the loss of one copy of a novel microRNA may lead to a novel disease mechanism.

The MCDR2 analysis did not involve use of Affymetrix data. A genetic mutation in *PROM1* had been previously identified and this gene was screened in a panel of MCDR2 patients to see if further variations within this gene occur. Though no new variations were found, three new families were identified that carry the c1,117C>T mutation in exon 10. These results are reported in Michaelides et al. (2010).

Finally, a significant proportion of the project was concerned with the analysis of the L/M gene arrays of BED families. Variations in these genes were identified as being the cause of the disorder and these novel results will be reported and published in the near future. Though the QPCR data were acceptable, improvements could be made by further optimisation of the reaction conditions and sourcing new DNA samples. Additional use of cloned plasmids containing known numbers of L or M genes would also have been beneficial for use as positive controls.

Overall, the analysis conducted has refined the loci of the inherited macular disorders MCDR3, MCDR4 and PBCRA with

extensive bioinformatic analysis for each identifying primary candidate genes for screening. CNV analysis identified a deletion and duplication event that segregates with SHFM and NCMD whilst mutation analysis revealed new MCDR2 families with *PROM1* mutations. Finally, the disease mechanism of Bornholm eye disease was determined.

13. APPENDICES

13.1 Appendix I

MCDR1 primers for failed screenings of *FBXL4* promoter and exon 1.

Figure 13.1 Primer binding regions for attempts at amplifying the *FBXL4* promoter and non-coding exon 1.

Table 13.1 Primers used for the attempts at screening *FBXL4* promoter and exon 1.

Primers	Name
CGATGGTCAAGAAGGATTCAT	Promoter F - NEW
ATTATTCCGAAGAGGATTTGTAGACACTA	NEW 1F
TGGCCTGGGAGCGGGCTGT	1F NEW 2
CTGTCCGTAGGACCCAGAGCAC	1F
GTCCAGACTCCCCAAAGCGCGGAG	1R
GAGGCACGTCAGCAGTTCTCAAGG	NEW 1R

13.2 Appendix II

MCDR1 6q disease region Affymetrix 50K genotyping data for all samples:

EB Genotype	EB Haplotype	LD	IM	KR	PB	WB	AJ	JH	AT	SK	db rsSNP ID	
AB	AB	BB	AB	NoCall	AB	AB	AB	AB	AA	AA	rs675974	
AB	AB	AA	AB	AA	AB	AA	AB	AA	BB	BB	rs595074	
BB	BB	NoCall	AA	AB	AB	AB	AB	BB	AB	AA	rs9320747	
AA	AA	NoCall	AA	AB	AB	AA	AA	AA	AA	AA	rs1933722	
AA	AA	AA	BB	NoCall	AB	AB	AB	AA	AB	BB	rs9320759	
AA	AA	AA	BB	AB	AB	AB	AB	AA	AB	BB	rs10484867	
AA	AA	AA	BB	NoCall	AB	AB	AB	AA	AB	BB	rs10484868	
AB	AA	AA	AA	BB	BB	AB	AB	AB	AB	AA	rs6926135	
AB	AA	AA	AA	BB	BB	AB	AA	AB	AB	AA	rs4557524	
AA	AA	AA	BB	AB	AB	AB	AB	NoCall	AB	BB	rs1872841	
AA	AA	AA	AA	AB	AB	AA	AA	AB	AA	AA	rs1487449	
AA	AA	NoCall	AA	AB	AB	NoCall	NoCall	NoCall	NoCall	NoCall	rs2029240	
AB	AB	NoCall	AA	NoCall	AB	AA	AB	BB	AA	AA	rs1376499	
AB	BB	NoCall	AA	AA	NoCall	NoCall	NoCall	NoCall	NoCall	NoCall	rs6911443	D6S1284, D6S1716, RP11-436D23.1
AB	BB	NoCall	AA	NoCall	AA	AA	AB	AB	AA	AA	rs10499017	
AA	AA	AA	AA	AA	AA	AA	AA	AB	AA	AA	rs10499018	
BB	BB	BB	AB	BB	AB	AB	AB	AB	BB	BB	rs150396	
AA	AA	AA	AB	AA	AB	AB	AB	AB	AA	AA	rs638458	
AB	AB	BB	BB	BB	AB	BB	BB	BB	AA	AB	rs6904416	RP3-453D15.1
AB	AB	AB	AB	AA	AA	AA	AA	AA	AA	AB	rs10499021	
AA	AA	NoCall	AB	NoCall	BB	BB	NoCall	BB	BB	AA	rs10499023	
AA	AA	AB	AA	AA	BB	AB	AA	AB	AA	AA	rs4131462	RP11-67P15.1
AB	BB	BB	BB	AB	BB	BB	BB	BB	BB	AB	rs10484608	POU3F2, FBXL4
AB	BB	BB	BB	NoCall	BB	NoCall	BB	NoCall	BB	AB	rs9321243	
AB	AA	AA	AA	AB	AA	AA	AA	AA	AA	AB	rs10484609	
BB	BB	NoCall	BB	BB	BB	BB	BB	BB	BB	BB	rs10484610	
AA	AA	NoCall	AA	AA	AA	AA	AA	AA	AA	AA	rs10484611	
AB	BB	BB	AB	AA	AB	NoCall	BB	AA	AA	AB	rs2029964	D6S1717, RP11-758C21.1
BB	BB	AB	AB	BB	AB	AA	AB	AB	AB	AB	rs4839746	C6orf168, COQ3, SFRS18, RP11-9819.4
BB	BB	BB	AB	AB	BB	BB	BB	BB	BB	BB	rs10499025	USP45
AA	AA	AA	AB	AB	AA	AA	AA	AA	AA	AA	rs6912892	RP1-199J3.3, Y_RNA
AB	AB	AB	BB	NoCall	BB	AB	AB	AB	BB	NoCall	rs2397341	CCNC, RP1-199J3.5, PRDM13
AA	AA	NoCall	AA	AB	AB	AA	AB	AA	AB	AB	rs10485222	
AB	AA	AB	AB	BB	NoCall	NoCall	AB	BB	NoCall	NoCall	rs9321649	Y_RNA
BB	BB	NoCall	AB	BB	NoCall	NoCall	BB	BB	NoCall	BB	rs518986	
AB	AB	AB	AA	NoCall	AA	AB	AA	BB	AA	NoCall	rs1552857	
BB	BB	BB	BB	AB	BB	BB	BB	BB	BB	BB	rs628428	
AA	AA	AA	AA	AA	AA	AA	AA	AA	AB	AA	rs9321691	
AB	AA	NoCall	AB	BB	NoCall	NoCall	NoCall	NoCall	NoCall	NoCall	rs10485226	
AA	AA	NoCall	AB	AB	AB	AB	AB	AA	BB	BB	rs10485227	
AB	BB	BB	AB	NoCall	AA	AA	AA	AB	AA	AB	rs10499028	
AB	AA	AA	AB	AB	BB	BB	BB	AB	BB	AB	rs10499026	MCHR2, RP11-1414.3, RP11-1414.2
BB	BB	NoCall	BB	BB	BB	BB	NoCall	BB	AB	BB	rs2474265	
AB	AB	AB	AB	AB	BB	AB	AB	NoCall	BB	BB	rs2474263	AL080285.1
BB	BB	AB	BB	BB	BB	BB	BB	BB	BB	AB	rs9321873	
AA	AA	AA	AA	AA	AA	AB	AA	AA	AA	AA	rs1012885	D6S1671, D6S475, RP3-344J20.2, RP3-344J20.2 & 1
BB	BB	BB	AB	AB	AB	AA	AB	AA	AA	AB	rs2073265	
AA	AA	AA	AB	AB	AB	AA	AA	AA	AB	AA	rs9321981	
BB	BB	NoCall	BB	BB	BB	AA	AB	AA	AB	AB	rs9321986	
AA	AA	AA	AB	NoCall	AB	NoCall	AA	AA	NoCall	AA	rs10499029	
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AA	AA	NoCall	AB	AB	AB	NoCall	AA	AA	AB	AA	rs1506087	
NoCall	AB	NoCall	NoCall	BB	NoCall	AA	AB	AA	AB	AB	rs1506089	
AA	AA	NoCall	AB	AB	NoCall	NoCall	NoCall	BB	BB	NoCall	rs2841308	
AA	AA	AA	AA	AA	AA	NoCall	AB	BB	BB	AB	rs2658133	
BB	BB	BB	BB	BB	BB	AB	NoCall	AA	AA	AB	rs1395117	
AA	AA	NoCall	AA	AA	AA	AB	AB	BB	BB	AB	rs2841300	
AA	AA	AA	AA	AB	AB	AA	AA	AA	AA	AA	rs9322033	SIM1
BB	BB	AB	NoCall	AB	AB	AB	BB	AB	BB	BB	rs718268	
BB	BB	BB	NoCall	AB	AB	NoCall	BB	NoCall	NoCall	NoCall	rs1395122	RP1-121G13.2
AA	AA	NoCall	AA	AB	AB	AA	AA	AA	AA	AA	rs3778033	
BB	BB	NoCall	BB	BB	BB	BB	BB	BB	AB	BB	rs240768	ASCC3
BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	rs240166	RP1-121G13.3
NoCall	AB	NoCall	AA	NoCall	AA	NoCall	NoCall	NoCall	AB	AA	rs10485139	
BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	NoCall	rs10485293	
AB	AA	AA	AB	AB	BB	AB	BB	AB	BB	BB	rs705608	
AA	AA	AA	AB	AB	BB	AA	BB	AB	AB	BB	rs4840159	
AA	AA	NoCall	AA	AA	AA	AB	AA	AA	AA	AA	rs10485300	
AB	BB	BB	AB	AB	AA	AB	AA	AB	AA	AA	rs817675	snoU13
BB	BB	BB	BB	AB	AA	BB	NoCall	AB	NoCall	NoCall	rs873649	
BB	BB	BB	BB	AB	AA	BB	AA	AB	AB	AA	rs2180338	
AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	rs10485266	
AB	AB	NoCall	NoCall	NoCall	AA	AA	AA	AA	AB	AA	rs549135	
AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	rs10485268	
BB	BB	BB	AB	AB	BB	BB	AB	BB	AB	BB	rs2255496	
AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	rs10485269	
BB	BB	NoCall	AB	AB	NoCall	BB	AB	BB	AB	NoCall	rs2248237	
AB	AA	AA	BB	AB	BB	NoCall	NoCall	BB	AB	BB	rs2224201	
AA	AA	AA	AB	AA	AA	AA	AA	AA	AB	AA	rs2852577	
AB	BB	BB	AB	BB	AA	BB	AB	AB	AB	AA	rs609531	
AB	AB	AA	AA	AA	AB	AA	AA	AA	AA	AA	rs596621	
BB	BB	NoCall	BB	BB	NoCall	AB	AB	AB	AB	AB	rs1856133	
AA	AA	AA	AA	AA	AA	AB	AB	AB	AB	AB	rs10485273	
AA	AA	AA	AA	AA	AA	NoCall	AB	AB	AB	AB	rs10485275	
AA	AA	NoCall	AA	AB	NoCall	NoCall	NoCall	NoCall	AB	BB	rs9322609	

13.3 Appendix III

MCDR3 5p15.33-p14.2 region 250K SNP data:

IV:14	V:7	V:8	III:4	IV:9	MCDR3 AD	dbSNP RS ID	Cytoband	IV:14	V:7	V:8	III:4	IV:9	MCDR3 AD	dbSNP RS ID	Cytoband
BB	BB	BB	BB	NoCall	BB	rs6555608	p15.33	BB	BB	BB	BB	BB	BB	rs17588991	p15.33
AA	AA	AA	AA	AA	AA	rs16900746	p15.33	BB	BB	BB	BB	NoCall	BB	rs2962041	p15.33
BB	BB	BB	BB	BB	BB	rs9312845	p15.33	AB	AB	AB	AB	AB	AB	rs4398676	p15.33
NoCall	AA	AA	AB	AB	AA	rs7717734	p15.33	AB	AB	AB	AB	NoCall	AB	rs7700579	p15.33
NoCall	NoCall	NoCall	AB	AB	AB	rs4956990	p15.33	AA	AA	AA	AA	AA	AA	rs7712396	p15.33
NoCall	BB	BB	AB	AB	BB	rs4956993	p15.33	BB	BB	BB	NoCall	NoCall	BB	rs2963266	p15.33
BB	AB	BB	AB	AB	BB	rs17559144	p15.33	NoCall	AA	NoCall	AA	NoCall	AA	rs10866513	p15.33
BB	AB	BB	AB	AB	BB	rs2864967	p15.33	AA	AA	AA	AA	AA	AA	rs16880246	p15.33
BB	NoCall	BB	BB	BB	BB	rs6878087	p15.33	AA	AA	AA	AA	AA	AA	rs17596634	p15.33
AA	NoCall	AA	AA	AA	AA	rs7714944	p15.33	AB	AB	AB	AB	NoCall	AB	rs1054540	p15.33
NoCall	BB	BB	BB	BB	BB	rs1541822	p15.33	AB	AB	AB	AB	BB	BB	rs4975681	p15.33
BB	BB	BB	BB	BB	BB	rs12173015	p15.33	AB	AB	BB	NoCall	BB	BB	rs6554915	p15.33
BB	BB	BB	BB	BB	BB	rs7713524	p15.33	NoCall	NoCall	NoCall	NoCall	NoCall	AB	rs10475076	p15.33
NoCall	BB	NoCall	BB	NoCall	BB	rs6555160	p15.33	AB	AB	AA	AA	AA	AA	rs4424035	p15.33
NoCall	AB	AA	AB	AA	AA	rs3734145	p15.33	NoCall	AB	NoCall	NoCall	NoCall	AB	rs6897438	p15.33
AA	AB	AA	AA	AA	AA	rs2721012	p15.33	BB	BB	BB	BB	BB	BB	rs10079800	p15.33
AA	NoCall	AA	AA	NoCall	AA	rs16900846	p15.33	BB	BB	BB	BB	BB	BB	rs6882694	p15.33
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NoCall	AA	NoCall	AA	NoCall	AA	rs2672762	p15.33	AA	AA	AA	AA	AA	AA	rs16883213	p15.33
BB	BB	BB	BB	NoCall	BB	rs6864453	p15.33	BB	NoCall	AB	NoCall	BB	BB	rs17599063	p15.33
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BB	AB	BB	BB	BB	BB	rs4956936	p15.33	BB	BB	BB	BB	BB	BB	rs16883787	p15.33
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BB	AB	BB	AB	AB	BB	rs2672757	p15.33	NoCall	AB	NoCall	NoCall	NoCall	AB	rs6895045	p15.33
AA	AB	AA	AB	AB	AA	rs2672734	p15.33	AB	AB	AB	AA	AA	AA	rs6554987	p15.33
AA	AB	AA	AB	AB	AA	rs2251843	p15.33	AB	NoCall	AB	BB	BB	BB	rs1392466	p15.33
BB	BB	BB	BB	NoCall	BB	rs1053226	p15.33	AB	NoCall	AA	AA	NoCall	AA	rs1392469	p15.33
AA	AA	AA	AA	NoCall	AA	rs2458814	p15.33	AB	AB	AB	BB	BB	BB	rs10040260	p15.33
BB	BB	BB	BB	BB	BB	rs3867180	p15.33	BB	BB	AB	BB	BB	BB	rs904756	p15.33
BB	NoCall	BB	BB	BB	BB	rs3853656	p15.33	BB	BB	NoCall	NoCall	AB	BB	rs16884579	p15.33
BB	BB	BB	BB	BB	BB	rs12521051	p15.33	AA	AB	AB	AA	AB	AA	rs5000660	p15.33
AA	AA	AA	AA	AA	AA	rs17563576	p15.33	AA	NoCall	AA	AA	AA	AA	rs904757	p15.33
NoCall	NoCall	NoCall	AA	AA	AA	rs3915384	p15.33	AA	AB	AA	AA	AA	AA	rs904758	p15.33
AA	NoCall	AA	AA	AA	AA	rs7726839	p15.33	BB	NoCall	NoCall	NoCall	NoCall	BB	rs9312955	p15.33
AB	AA	AB	AB	AA	AA	rs1617985	p15.33	BB	BB	BB	BB	NoCall	BB	rs7711522	p15.33
NoCall	BB	AB	AB	BB	BB	rs1709531	p15.33	BB	AB	AB	BB	BB	BB	rs4975845	p15.33
NoCall	AA	AB	AB	AB	AA	rs1697985	p15.33	AA	AB	AB	AA	AA	AA	rs7702843	p15.33
NoCall	NoCall	NoCall	NoCall	NoCall	AB	rs4957080	p15.33	BB	BB	BB	BB	BB	BB	rs4588629	p15.33
AB	BB	AB	AB	AB	BB	rs1697976	p15.33	AA	AA	AA	AA	AA	AA	rs10085011	p15.33
AA	AA	AA	AA	AA	AA	rs6862648	p15.33	AA	AA	AA	NoCall	AA	AA	rs4304110	p15.33
NoCall	AB	BB	AB	AB	BB	rs6555409	p15.33	NoCall	AB	BB	BB	NoCall	BB	rs4975731	p15.33
NoCall	AA	NoCall	NoCall	NoCall	AA	rs445300	p15.33	AA	AA	AA	AA	AA	AA	rs17535721	p15.33
AA	AA	AA	AA	AA	AA	rs9313187	p15.33	BB	BB	BB	BB	BB	BB	rs10078791	p15.33
NoCall	NoCall	AA	NoCall	AB	AA	rs429739	p15.33	NoCall	AB	NoCall	AA	NoCall	AA	rs4975732	p15.33
NoCall	NoCall	BB	NoCall	AB	BB	rs445674	p15.33	BB	BB	BB	BB	BB	BB	rs6875087	p15.33
NoCall	AA	NoCall	NoCall	NoCall	AA	rs6859411	p15.33	AA	NoCall	AA	AA	NoCall	AA	rs6555013	p15.33
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BB	NoCall	BB	BB	NoCall	BB	rs7711616	p15.33	AA	AA	AA	AA	AA	AA	rs16901230	p15.33
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AB	AA	AA	AA	AA	AA	rs6555556	p15.33	AA	NoCall	AA	AA	NoCall	AA	rs1532272	p15.33
AA	NoCall	AB	AA	AA	AA	rs13179524	p15.33	AA	AA	AA	AA	AA	AA	rs4975744	p15.33
AA	AA	AA	AA	AA	AA	rs16903688	p15.33	NoCall	AA	AB	NoCall	NoCall	AA	rs1684969	p15.33
BB	NoCall	BB	BB	NoCall	BB	rs16868701	p15.33	NoCall	NoCall	AB	AB	NoCall	AB	rs1689747	p15.33
NoCall	BB	BB	BB	BB	BB	rs4975533	p15.33	AA	AA	AB	AA	AA	AA	rs2353580	p15.33
AA	AA	AA	AA	AB	AA	rs10071261	p15.33	AA	AB	AA	AB	AB	AA	rs883823	p15.33
AA	AA	NoCall	AA	AA	AA	rs4975569	p15.33	AA	AA	AA	AA	AA	AA	rs4975752	p15.33
BB	NoCall	NoCall	NoCall	NoCall	BB	rs4365871	p15.33	BB	AB	BB	BB	BB	BB	rs260406	p15.33
AA	AB	AB	AA	AB	AA	rs2334955	p15.33	BB	BB	AB	BB	BB	BB	rs17537048	p15.33
AB	BB	BB	AB	BB	BB	rs4975552	p15.33	AA	AB	AA	AB	AA	AA	rs10042507	p15.33
AA	AA	AA	AA	AA	AA	rs12653975	p15.33	AA	AB	NoCall	NoCall	AA	AA	rs11744276	p15.33
BB	NoCall	BB	BB	BB	BB	rs11948939	p15.33	AB	AB	BB	AB	BB	BB	rs7737997	p15.33
NoCall	AA	AA	NoCall	AA	AA	rs6892881	p15.33	AB	AB	NoCall	AB	AB	AB	rs4975775	p15.33
AB	NoCall	AA	AA	NoCall	AA	rs11951820	p15.33	BB	AB	NoCall	AB	AB	BB	rs1123381	p15.33
AB	AA	AA	NoCall	AB	AA	rs11133652	p15.33	AB	AB	BB	AB	NoCall	BB	rs1123391	p15.33
BB	NoCall	BB	BB	NoCall	BB	rs6554633	p15.33	AB	AB	AA	AB	AB	AA	rs1123390	p15.33
BB	BB	AB	BB	AB	BB	rs4246740	p15.33	AB	BB	BB	BB	BB	BB	rs10043083	p15.33
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NoCall	BB	NoCall	NoCall	NoCall	BB	rs4975596	p15.33	AA	NoCall	AB	AA	AA	AA	rs746365	p15.33
AA	NoCall	AA	AA	AA	AA	rs7736074	p15.33	NoCall	AB	NoCall	AB	AB	AB	rs11747860	p15.33
BB	BB	BB	BB	BB	BB	rs10060236	p15.33	AA	AA	AB	AB	AB	AA	rs13190016	p15.33
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BB	BB	AB	AB	BB	BB	rs7445640	p15.33	AB	BB	AB	AB	AB	BB	rs11952498	p15.33
BB	BB	BB	BB	BB	BB	rs6554677	p15.33	AA	AA	AA	AB	AA	AA	rs11960536	p15.33
NoCall	NoCall	NoCall	NoCall	NoCall	AB	rs4975621	p15.33	AB	AB	AB	AA	AB	AA	rs7448302	p15.33
BB	AB	NoCall	BB	BB	BB	rs6554679	p15.33	AA	AA	AA	AA	AA	AA	rs11133930	p15.33
AA	NoCall	AA	AA	AA	AA	rs12513872	p15.33	AA	AA	AA	AA	AA	AA	rs16903364	p15.33
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AB	AA	AA	NoCall	AB	AA	rs2853668	p15.33	BB	BB	BB	BB	BB	BB	rs16903407	p15.33
NoCall	BB	NoCall	NoCall	NoCall	BB	rs2735845	p15.33	AB	AA	AA	AB	AA	AA	rs13171126	p15.33
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AB	AA	AA	AB	AA	AA	rs465498	p15.33	AB	BB	NoCall	NoCall	BB	BB	rs3923314	p15.33
AB	NoCall	AA	AB	AA	AA	rs37008	p15.33	AB	NoCall	BB	AB	AB	BB	rs4866625	p15.33
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AB	BB	AB	AB	AB	BB	rs4975646	p15.33	AA	AA	AA	AA	AA	AA	rs6861416	p15.33
NoCall	AA	AB	AA	AB	AA	rs420422	p15.33	NoCall	NoCall	AA	NoCall	NoCall	AA	rs16903629	p15.33
BB	BB	AB	AB	BB	BB	rs2937639	p15.33	AA	AB	AA	AA	AA	AA	rs10041522	p15.33
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BB	BB	BB	BB	BB	BB	rs17588991	p15.33	AB	AB	AB	AB	AB	AB	rs117622	p15.33
								AA	AA	AA	AA	AA	AA	rs316589	p15.33
								AA	AA	AA	AA	AA	AA	rs898061	p15.33

IV:14	V:7	V:8	III:4	IV:9	MCDR3 AD	dbSNP RS ID	Cytoband	IV:14	V:7	V:8	III:4	IV:9	MCDR3 AD	dbSNP RS ID	Cytoband
AA	AA	AA	AA	AA	AA	rs898061	p15.33	AB	AA	AB	AB	AB	AA	rs10044519	p15.33
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BB	AB	AB	BB	AB	BB	rs2628163	p15.33	NoCall	AA	AA	AB	NoCall	AA	rs13160491	p15.33
AA	AB	AB	AA	AB	AA	rs13169221	p15.33	NoCall	BB	BB	AB	AA	Exclude	rs2934551	p15.33
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BB	BB	NoCall	BB	NoCall	BB	rs16869166	p15.33	NoCall	AB	AB	AB	AB	AB	rs10475186	p15.33
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AB	AB	AB	AB	AB	AB	rs4866643	p15.33	BB	NoCall	BB	NoCall	NoCall	BB	rs4270702	p15.33
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BB	BB	BB	BB	BB	BB	rs16869189	p15.33	NoCall	NoCall	BB	BB	BB	BB	rs2974660	p15.33
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BB	AB	AB	BB	AB	BB	rs11744790	p15.33	AA	AA	NoCall	AA	AA	AA	rs7727778	p15.33
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BB	AB	AB	BB	AB	BB	rs11748913	p15.33	AA	AA	AB	AA	AA	AA	rs457045	p15.33
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AA	AA	AA	AA	AA	AA	rs16869936	p15.33	NoCall	BB	AB	AB	AB	BB	rs17620715	p15.33
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IV:14	V:7	V:8	III:4	IV:9	MCDR3 AD	dbSNP RS ID	Cytoband	IV:14	V:7	V:8	III:4	IV:9	MCDR3 AD	dbSNP RS ID	Cytoband
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BB	NoCall	BB	BB	NoCall	BB	rs1867708	p15.33	BB	BB	AB	AB	AB	BB	rs261179	p15.33
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AA	AA	AA	AB	AA	AA	rs7703450	p15.33	BB	NoCall	NoCall	BB	NoCall	BB	rs10061612	p15.32
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AA	NoCall	AA	AA	AA	AA	rs2398624	p15.33	NoCall	AA	AA	AA	NoCall	AA	rs1469468	p15.32
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AB	AA	AA	AA	AA	AA	rs13180582	p15.33	AA	AA	AA	AA	AA	AA	rs868647	p15.32
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IV:14	V:7	V:8	III:4	IV:9	MCDR3 AD	dbSNP RS ID	Cytoband	IV:14	V:7	V:8	III:4	IV:9	MCDR3 AD	dbSNP RS ID	Cytoband
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IV:14	V:7	V:8	III:4	IV:9	MCDR3 AD	dbSNP RS ID	Cytoband	IV:14	V:7	V:8	III:4	IV:9	MCDR3 AD	dbSNP RS ID	Cytoband
AA	NoCall	AA	AA	NoCall	AA	rs1346548	p15.31	NoCall	NoCall	NoCall	NoCall	NoCall	AB	rs13163920	p15.31
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BB	NoCall	NoCall	NoCall	NoCall	BB	rs16876997	p15.31	AA	AA	NoCall	NoCall	AB	AA	rs11956948	p15.31
BB	BB	NoCall	BB	BB	BB	rs12109930	p15.31	BB	AB	BB	BB	BB	BB	rs450302	p15.31
BB	NoCall	AB	BB	BB	BB	rs2937785	p15.31	NoCall	BB	BB	BB	NoCall	BB	rs274645	p15.31
AA	AB	AB	AB	AB	AA	rs1875541	p15.31	NoCall	AA	NoCall	AA	NoCall	AA	rs12109729	p15.31
NoCall	AA	AB	AA	AA	AA	rs2964734	p15.31	AA	AB	AA	AA	AB	AA	rs275442	p15.31
AB	BB	BB	BB	BB	BB	rs7727548	p15.31	AB	AB	NoCall	AB	AB	AB	rs275481	p15.31
AB	AB	AB	AB	AB	AB	rs1037687	p15.31	BB	AB	AB	BB	AB	BB	rs275480	p15.31
NoCall	AB	AA	AB	AB	AA	rs7726513	p15.31	BB	NoCall	BB	BB	BB	BB	rs11134178	p15.31
AA	AB	AA	AB	AB	AA	rs7726521	p15.31	NoCall	AB	AA	AA	AA	AA	rs275467	p15.31
BB	BB	BB	BB	BB	BB	rs16877061	p15.31	NoCall	AA	AA	AB	AA	AA	rs11749640	p15.31
AA	AB	AA	AB	AB	AA	rs11134150	p15.31	BB	NoCall	BB	BB	BB	BB	rs17181935	p15.31
BB	AB	BB	AB	AB	BB	rs6885691	p15.31	BB	BB	AB	BB	BB	BB	rs13160728	p15.31
AA	AB	AB	AB	NoCall	AA	rs753426	p15.31	NoCall	BB	BB	BB	BB	BB	rs16878072	p15.31
NoCall	AB	AA	AB	AB	AA	rs4701718	p15.31	AA	NoCall	AA	AA	AA	AA	rs12189140	p15.31
AA	AB	AB	AB	AB	AA	rs2964761	p15.31	NoCall	BB	BB	BB	BB	BB	rs11134182	p15.31
AB	BB	AB	BB	AB	BB	rs7718918	p15.31	NoCall	AA	NoCall	NoCall	NoCall	AA	rs12514931	p15.31
AB	AB	AB	AB	AB	AB	rs1551572	p15.31	AA	AA	AA	AA	AA	AA	rs10512890	p15.31
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BB	BB	BB	BB	BB	BB	rs17825562	p15.31	AA	AA	AA	AA	AA	AA	rs903867	p15.31
AB	NoCall	NoCall	NoCall	NoCall	AB	rs12520404	p15.31	NoCall	BB	BB	NoCall	NoCall	BB	rs903866	p15.31
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BB	AB	BB	BB	BB	BB	rs13189546	p15.31	BB	BB	BB	BB	NoCall	BB	rs11134188	p15.31
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NoCall	AB	NoCall	BB	NoCall	BB	rs271415	p15.31	NoCall	BB	NoCall	BB	NoCall	BB	rs736095	p15.31
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BB	NoCall	BB	BB	BB	BB	rs10035149	p15.31	NoCall	AB	AA	AA	AA	AA	rs4702400	p15.31
AA	AA	AA	AB	AA	AA	rs271399	p15.31	AB	AB	AA	AA	AA	AA	rs17804457	p15.31
AB	AB	BB	BB	BB	BB	rs12652912	p15.31	AA	AA	AA	NoCall	AA	AA	rs903863	p15.31
NoCall	BB	BB	AB	BB	BB	rs17775764	p15.31	AB	AB	BB	BB	BB	BB	rs4702401	p15.31
AB	NoCall	BB	BB	BB	BB	rs10462815	p15.31	NoCall	NoCall	AA	NoCall	AA	AA	rs7722047	p15.31
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AB	BB	BB	NoCall	BB	BB	rs16877286	p15.31	AB	AB	BB	BB	BB	BB	rs10512893	p15.31
AA	AA	AA	AA	AA	AA	rs4702348	p15.31	AB	AB	BB	NoCall	NoCall	BB	rs11134192	p15.31
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AB	AB	AA	AA	NoCall	AA	rs10512858	p15.31	NoCall	BB	NoCall	BB	NoCall	BB	rs6896984	p15.31
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AB	NoCall	NoCall	BB	NoCall	BB	rs824632	p15.31	AB	AB	BB	BB	BB	BB	rs6869714	p15.31
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BB	NoCall	AB	BB	NoCall	BB	rs10044719	p15.31	AA	AB	NoCall	AA	AB	AA	rs10056446	p15.31
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AA	AA	AA	AA	AA	AA	rs11948869	p15.31	AB	NoCall	BB	BB	NoCall	BB	rs10462830	p15.31
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AA	AA	NoCall	AA	AA	AA	rs16877524	p15.31	NoCall	BB	NoCall	NoCall	BB	BB	rs1496362	p15.31
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AA	NoCall	AA	AA	AA	AA	rs16877663	p15.31	AA	NoCall	AA	AA	AA	AA	rs11134211	p15.31
AB	AA	AA	AB	AA	AA	rs3776439	p15.31	AB	AB	BB	AB	AB	BB	rs11959795	p15.31
BB	BB	AB	BB	BB	BB	rs6882580	p15.31	AA	AA	AB	AA	AA	AA	rs2202445	p15.31
AB	BB	BB	AB	BB	BB	rs12374467	p15.31	AB	AB	AA	AB	AB	AA	rs2397355	p15.31
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AA	NoCall	AA	AA	AA	AA	rs8192180	p15.31	AA	AA	AA	AB	AB	AA	rs10512921	p15.31
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AA	AA	AA	AA	AA	AA	rs523854	p15.31	BB	AB	BB	NoCall	BB	BB	rs1864071	p15.31
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AB	AB	BB	AB	AB	BB	rs10512874	p15.31	AB	AA	AB	AA	AB	AA	rs11134233	p15.31
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BB	AB	NoCall	AB	BB	BB	rs274719	p15.31	AA	NoCall	AA	AA	AA	AA	rs7714705	p15.31
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AA	AA	AB	AA	AA	AA	rs2279653	p15.31	AB	BB	BB	AB	AB	BB	rs6880956	p15.31
AA	AB	AB	AB	AA	AA	rs274678	p15.31	AB	AA	AA	AB	AB	AA	rs13174252	p15.31
NoCall	NoCall	NoCall	NoCall	NoCall	AB	rs13163920	p15.31	NoCall	NoCall	NoCall	NoCall	AA	AA	rs4701786	p15.31

IV:14	V:7	V:8	III:4	IV:9	MCDR3 AD	dbSNP RS ID	Cytoband	IV:14	V:7	V:8	III:4	IV:9	MCDR3 AD	dbSNP RS ID	Cytoband
NoCall	NoCall	NoCall	NoCall	AA	AA	rs4701786	p15.31	AB	AB	AB	AB	AB	AB	rs6892570	p15.2
AA	AA	AA	AA	AA	AA	rs4235580	p15.31	BB	BB	BB	BB	BB	BB	rs16880162	p15.2
AA	AA	AA	AA	AA	AA	rs7734088	p15.31	NoCall	BB	NoCall	NoCall	BB	BB	rs6864857	p15.2
AA	AB	AA	AB	AA	AA	rs4524477	p15.31	AB	BB	BB	BB	BB	BB	rs4702544	p15.2
AB	AB	AB	NoCall	AB	AB	rs11134242	p15.31	AB	AA	AA	AA	AA	AA	rs7725925	p15.2
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AB	AB	BB	AB	NoCall	BB	rs12152969	p15.31	NoCall	AB	AB	AB	AB	AB	rs2388624	p15.2
NoCall	BB	BB	BB	BB	BB	rs16878822	p15.31	AA	AB	AB	AB	AB	AA	rs9313234	p15.2
BB	BB	BB	BB	BB	BB	rs2914290	p15.31	AA	AB	AB	AB	AB	AA	rs873231	p15.2
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BB	BB	BB	BB	BB	BB	rs16879182	p15.31	AA	NoCall	NoCall	NoCall	NoCall	AA	rs341876	p15.2
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AB	BB	NoCall	BB	NoCall	BB	rs6863183	p15.31	BB	BB	BB	BB	BB	BB	rs13356951	p15.2
AB	AA	AA	AA	AA	AA	rs6863763	p15.31	AA	AA	AA	AB	AB	AA	rs2963394	p15.2
AB	NoCall	BB	BB	NoCall	BB	rs7730131	p15.31	NoCall	BB	BB	AB	BB	BB	rs1158727	p15.2
AB	NoCall	NoCall	NoCall	NoCall	AB	rs4401546	p15.31	NoCall	AA	NoCall	NoCall	NoCall	AA	rs995553	p15.2
BB	BB	BB	BB	BB	BB	rs11134269	p15.31	AB	BB	BB	AB	BB	BB	rs13182390	p15.2
BB	AB	AB	BB	BB	BB	rs6866169	p15.31	AB	BB	BB	AB	BB	BB	rs12653298	p15.2
AB	AB	AB	AA	AA	AA	rs7702491	p15.31	AB	NoCall	AB	NoCall	NoCall	AB	rs4702591	p15.2
AA	AB	AB	AA	AA	AA	rs2968311	p15.31	BB	BB	BB	BB	BB	BB	rs4280839	p15.2
AA	AA	AA	AA	AA	AA	rs17195685	p15.31	NoCall	NoCall	AB	AB	NoCall	AB	rs16881529	p15.2
NoCall	BB	NoCall	NoCall	NoCall	BB	rs17268677	p15.31	AA	NoCall	AA	NoCall	NoCall	AA	rs4074122	p15.2
AA	AA	AA	AA	AA	AA	rs13166043	p15.31	AA	AB	AB	AB	AA	AA	rs2962694	p15.2
AA	AA	AA	NoCall	AA	AA	rs13164870	p15.31	BB	NoCall	BB	BB	BB	BB	rs10512994	p15.2
AA	AB	AB	AB	AA	AA	rs4615273	p15.31	AB	AB	AB	AB	AB	AB	rs11955530	p15.2
NoCall	NoCall	NoCall	BB	BB	BB	rs13189965	p15.31	BB	AB	AB	AB	BB	BB	rs2963341	p15.2
AA	NoCall	AA	AA	AA	AA	rs11747216	p15.31	AA	AA	AA	AA	AA	AA	rs16881658	p15.2
AA	AA	AA	AA	AA	AA	rs1023853	p15.31	AB	AB	AB	AB	NoCall	AB	rs9313258	p15.2
AB	AB	AB	AB	AA	AA	rs1453360	p15.31	AA	AB	AB	AB	NoCall	AA	rs2963324	p15.2
AA	AA	AA	AA	AA	AA	rs1453359	p15.31	AA	AB	AB	NoCall	AA	AA	rs4495147	p15.2
NoCall	AA	AA	AA	AA	AA	rs1530962	p15.31	AA	AB	AB	AB	AA	AA	rs13359910	p15.2
NoCall	AB	AB	AA	AA	AA	rs16879792	p15.31	NoCall	AB	AB	AB	NoCall	AB	rs6555588	p15.2
AA	AA	AA	AA	AA	AA	rs9313221	p15.31	AA	AB	AB	AB	AA	AA	rs1124255	p15.2
AB	AB	AB	AB	AB	AB	rs1597956	p15.31	NoCall	AB	AB	AB	BB	BB	rs4612031	p15.2
BB	NoCall	AB	AB	NoCall	BB	rs34799743	p15.31	AB	AA	AA	AA	AA	AA	rs1350379	p15.2
BB	BB	BB	BB	BB	BB	rs2658160	p15.2	AB	AB	AB	AB	BB	BB	rs2962720	p15.2
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BB	AB	AB	BB	NoCall	BB	rs6555524	p15.2	NoCall	BB	BB	AB	BB	BB	rs181939	p15.2
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BB	BB	BB	BB	BB	BB	rs7701145	p15.2	AB	AA	AA	AA	AA	AA	rs707635	p15.2
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NoCall	BB	BB	NoCall	NoCall	BB	rs10780108	p15.2	NoCall	NoCall	BB	BB	BB	BB	rs786848	p15.2
AB	AB	AB	AB	AB	AB	rs6892570	p15.2	AB	BB	NoCall	BB	NoCall	BB	rs1505059	p15.2

IV:14	V:7	V:8	III:4	IV:9	MCDR3 AD	dbSNP RS ID	Cytoband	IV:14	V:7	V:8	III:4	IV:9	MCDR3 AD	dbSNP RS ID	Cytoband
AB	BB	NoCall	BB	NoCall	BB	rs1505059	p15.2	AA	AA	AA	AA	AA	AA	rs10513046	p15.2
BB	AB	BB	BB	BB	BB	rs253645	p15.2	BB	BB	NoCall	BB	BB	BB	rs11745136	p15.2
BB	AB	BB	BB	BB	BB	rs253646	p15.2	NoCall	NoCall	NoCall	NoCall	AB	AB	rs1858845	p15.2
NoCall	AA	NoCall	AA	NoCall	AA	rs16881976	p15.2	AA	AA	AA	AA	AB	AA	rs3094355	p15.2
AA	AA	AA	AA	AA	AA	rs253657	p15.2	NoCall	BB	BB	BB	BB	BB	rs2387178	p15.2
NoCall	NoCall	AA	AA	NoCall	AA	rs2619913	p15.2	AA	NoCall	AA	AA	AA	AA	rs3110987	p15.2
BB	BB	BB	BB	BB	BB	rs2619911	p15.2	BB	BB	BB	BB	AB	BB	rs3110986	p15.2
AB	NoCall	BB	BB	AB	BB	rs1479648	p15.2	NoCall	AA	NoCall	AA	NoCall	AA	rs16883520	p15.2
NoCall	NoCall	NoCall	NoCall	NoCall	AB	rs10062083	p15.2	BB	BB	BB	BB	AB	BB	rs2961874	p15.2
AB	AB	AA	AA	AB	AA	rs6863413	p15.2	AA	AA	AA	AA	AB	AA	rs2067848	p15.2
AB	AB	BB	BB	AB	BB	rs899575	p15.2	BB	AB	BB	AB	AB	BB	rs11134371	p15.2
NoCall	AB	BB	BB	AB	BB	rs899576	p15.2	NoCall	AB	AA	AB	AB	AA	rs882142	p15.2
NoCall	NoCall	NoCall	AA	NoCall	AA	rs886511	p15.2	AA	AA	AA	AA	AA	AA	rs10067092	p15.2
AA	AA	AA	AA	AA	AA	rs1806147	p15.2	BB	AB	BB	AB	BB	BB	rs4263482	p15.2
AA	AA	AA	AA	AA	AA	rs886510	p15.2	NoCall	AB	NoCall	AB	NoCall	AB	rs7446935	p15.2
AA	AB	AA	AA	AB	AA	rs2526122	p15.2	AA	AB	AA	AB	AA	AA	rs13159193	p15.2
AA	AB	AA	AA	AB	AA	rs2256569	p15.2	AB	AB	AB	AB	AB	AB	rs17196719	p15.2
AB	AA	NoCall	AA	AA	AA	rs3777276	p15.2	NoCall	AB	AA	NoCall	NoCall	AA	rs10491233	p15.2
AB	NoCall	BB	BB	BB	BB	rs3777277	p15.2	BB	AB	BB	AB	BB	BB	rs12517509	p15.2
AB	AA	NoCall	NoCall	NoCall	AA	rs3822773	p15.2	NoCall	AA	NoCall	AA	AA	AA	rs886103	p15.2
AB	AA	AA	AA	AA	AA	rs10039094	p15.2	BB	AB	BB	AB	BB	BB	rs2387171	p15.2
AB	BB	BB	BB	BB	BB	rs3797933	p15.2	AA	AA	AA	AA	NoCall	AA	rs16883661	p15.2
BB	BB	BB	AB	BB	BB	rs3777281	p15.2	NoCall	AB	NoCall	AB	NoCall	AB	rs886101	p15.2
AA	AA	AA	AA	AB	AA	rs877191	p15.2	BB	AB	BB	AB	BB	BB	rs982390	p15.2
AA	AA	AA	AA	AA	AA	rs877190	p15.2	AB	AB	AB	AB	BB	BB	rs4701850	p15.2
AB	AB	AB	AB	AB	AB	rs40669	p15.2	BB	NoCall	BB	AB	BB	BB	rs2963899	p15.2
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BB	AB	BB	BB	BB	BB	rs40662	p15.2	BB	BB	BB	BB	NoCall	BB	rs7707030	p15.2
BB	BB	BB	BB	BB	BB	rs1806018	p15.2	NoCall	BB	BB	BB	NoCall	BB	rs7707194	p15.2
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AA	NoCall	AA	AA	AA	AA	rs1805981	p15.2	AA	AA	AB	AA	AA	AA	rs17354739	p15.2
AA	AB	NoCall	AA	AA	AA	rs40709	p15.2	NoCall	NoCall	NoCall	NoCall	NoCall	AB	rs1428958	p15.2
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AB	BB	BB	BB	BB	BB	rs13162749	p15.2	AB	AB	BB	AB	AB	BB	rs1428949	p15.2
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BB	BB	NoCall	BB	NoCall	BB	rs767439	p15.2	AA	AA	AA	AA	AA	AA	rs1428950	p15.2
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BB	NoCall	NoCall	BB	BB	BB	rs3777312	p15.2	NoCall	AB	BB	AB	AB	BB	rs7716386	p15.2
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BB	BB	BB	BB	BB	BB	rs3777324	p15.2	AA	AA	AB	AA	AA	AA	rs38086	p15.2
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AB	BB	BB	BB	BB	BB	rs2696371	p15.2	AB	AB	NoCall	AB	AB	AB	rs40233	p15.2
AB	AA	AA	AA	AA	AA	rs1918778	p15.2	AB	NoCall	NoCall	NoCall	NoCall	AB	rs17196866	p15.2
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BB	AB	BB	BB	BB	BB	rs4702625	p15.2	BB	BB	BB	BB	BB	BB	rs314736	p15.2
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AA	AA	AA	NoCall	NoCall	AA	rs3756727	p15.2	AA	NoCall	AA	AA	AA	AA	rs6554566	p15.2
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AB	AB	AA	AA	AB	AA	rs6882326	p15.2	BB	BB	BB	AB	AB	BB	rs6884905	p15.2
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AA	AA	AA	AA	AA	AA	rs10513046	p15.2	AB	BB	BB	BB	BB	BB	rs2578619	p15.2

IV:14	V:7	V:8	III:4	IV:9	MCDR3 AD	dbSNP RS ID	Cytoband	IV:14	V:7	V:8	III:4	IV:9	MCDR3 AD	dbSNP RS ID	Cytoband
AB	BB	BB	BB	BB	BB	rs2578619	p15.2	AA	AA	AA	AA	AA	AA	rs10474907	p15.2
AA	AA	AA	AA	AA	AA	rs2292267	p15.2	AB	AB	AB	NoCall	NoCall	AB	rs706234	p15.2
AB	AA	AA	NoCall	NoCall	AA	rs2244960	p15.2	AA	NoCall	AA	AA	AA	AA	rs1024618	p15.2
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AB	BB	NoCall	BB	BB	BB	rs679416	p15.2	AB	AB	AB	AB	AB	AB	rs2907096	p15.2
AA	AB	AA	AA	AA	AA	rs555521	p15.2	AB	AB	AB	AB	AB	AB	rs2400006	p15.2
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AA	NoCall	NoCall	NoCall	NoCall	AA	rs4702690	p15.2	NoCall	NoCall	NoCall	NoCall	NoCall	AB	rs16901233	p15.2
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BB	BB	BB	BB	BB	BB	rs2589661	p15.2	BB	AB	AB	AB	AB	BB	rs10073056	p15.2
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BB	BB	AB	BB	AB	BB	rs814586	p15.2	AB	BB	AB	BB	NoCall	BB	rs2036319	p15.2
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AB	NoCall	AB	AB	AB	AB	rs1107818	p15.2	NoCall	AA	NoCall	AA	NoCall	AA	rs2040648	p15.2
BB	AB	AB	AB	BB	BB	rs34091808	p15.2	AA	AA	AA	AA	AA	AA	rs32138	p15.2
AA	NoCall	NoCall	AA	AA	AA	rs16898812	p15.2	AA	AA	AA	AA	AA	AA	rs16901522	p15.2
NoCall	AA	NoCall	AA	NoCall	AA	rs5745308	p15.2	BB	BB	BB	BB	BB	BB	rs1076202	p15.2
BB	BB	BB	BB	BB	BB	rs3797119	p15.2	AA	AA	AA	AA	AA	AA	rs32121	p15.2
AA	AA	AA	AA	AA	AA	rs5745254	p15.2	BB	AB	BB	AB	BB	BB	rs10513097	p15.2
BB	BB	BB	BB	BB	BB	rs5745250	p15.2	BB	BB	BB	BB	BB	BB	rs6554624	p15.2
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AA	AA	AA	NoCall	AA	AA	rs267977	p15.2	AA	AA	NoCall	AA	NoCall	AA	rs10474920	p15.2
AB	BB	BB	BB	AB	BB	rs267973	p15.2	AA	AA	AA	AA	AA	AA	rs12518049	p15.2
BB	BB	BB	NoCall	NoCall	BB	rs413846	p15.2	BB	BB	BB	BB	BB	BB	rs7725841	p15.2
AA	AA	AA	AA	AA	AA	rs4701880	p15.2	AA	AA	AB	AA	AA	AA	rs6554627	p15.2
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BB	BB	BB	BB	BB	BB	rs267950	p15.2	AA	AB	AA	AA	AA	AA	rs11133654	p15.2
AB	AB	AB	AB	NoCall	AB	rs267925	p15.2	BB	BB	BB	BB	BB	BB	rs25950	p15.2
AA	AB	AA	AA	AA	AA	rs2964798	p15.2	NoCall	AA	AA	AA	NoCall	AA	rs17216753	p15.2
BB	NoCall	BB	BB	NoCall	BB	rs16901038	p15.2	AA	AB	AB	AA	AA	AA	rs26148	p15.2
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AB	BB	NoCall	AB	NoCall	BB	rs12716076	p15.2	AA	NoCall	NoCall	AA	AA	AA	rs32432	p15.2
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AB	BB	BB	AB	AB	BB	rs11740905	p15.2	BB	BB	BB	AB	BB	BB	rs12659996	p15.2
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BB	AB	BB	AB	AB	BB	rs4361487	p15.2	BB	NoCall	BB	BB	NoCall	BB	rs16901779	p15.2
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AA	AB	AA	NoCall	NoCall	AA	rs4352573	p15.2	AB	AB	AB	AB	AA	AA	rs4312856	p15.2
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AA	AB	AB	NoCall	AB	AA	rs11948902	p15.2	AB	AB	AB	AB	BB	BB	rs6889997	p15.2
BB	AB	AB	AB	BB	BB	rs7705870	p15.2	BB	BB	BB	BB	BB	BB	rs1428513	p15.2
AA	AB	AA	AB	NoCall	AA	rs1860245	p15.2	AA	AA	AB	AA	AA	AA	rs2245050	p15.2
BB	BB	AB	BB	BB	BB	rs10491237	p15.2	AB	AB	AB	AB	NoCall	AB	rs2727592	p15.2
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BB	BB	BB	BB	BB	BB	rs16901146	p15.2	NoCall	BB	BB	BB	NoCall	BB	rs4357035	p15.2
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NoCall	NoCall	NoCall	AA	NoCall	AA	rs16901216	p15.2	AB	NoCall	AB	BB	NoCall	BB	rs1870329	p15.2
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AA	AA	AA	AA	AA	AA	rs10474907	p15.2	NoCall	BB	BB	NoCall	NoCall	BB	rs1458477	p15.2

IV:14	V:7	V:8	III:4	IV:9	MCDR3 AD	dbSNP RS ID	Cytoband	IV:14	V:7	V:8	III:4	IV:9	MCDR3 AD	dbSNP RS ID	Cytoband
NoCall	BB	BB	NoCall	NoCall	BB	rs1458477	p15.2	NoCall	AB	AB	AA	AA	AA	rs1867679	p15.2
AB	BB	AB	NoCall	BB	BB	rs1542428	p15.2	BB	BB	BB	BB	BB	BB	rs1867678	p15.2
AB	BB	AB	BB	BB	BB	rs1379901	p15.2	AA	AB	AB	NoCall	AA	AA	rs16902790	p15.2
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AA	AA	AA	NoCall	AA	AA	rs6554662	p15.2	NoCall	AB	NoCall	BB	NoCall	BB	rs6896894	p15.2
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AB	AB	AB	AA	AA	AA	rs12517202	p15.2	BB	AB	AB	BB	BB	BB	rs895321	p15.2
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AA	AA	AA	AA	AA	AA	rs2400141	p15.2	AA	AB	NoCall	AA	AA	AA	rs895316	p15.2
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BB	BB	BB	BB	BB	BB	rs2968105	p15.2	AA	AA	AA	AA	AA	AA	rs895314	p15.2
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BB	BB	NoCall	BB	BB	BB	rs10075629	p15.2	AB	AA	AA	AA	AB	AA	rs795526	p15.2
AA	AA	AA	AA	NoCall	AA	rs10073609	p15.2	AB	AB	AB	AB	AB	AB	rs795525	p15.2
AA	AA	AA	AA	AA	AA	rs6891532	p15.2	BB	NoCall	AB	AB	BB	BB	rs1900161	p15.2
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AA	AA	AB	AA	NoCall	AA	rs10035331	p15.2	AA	AA	AA	AA	AA	AA	rs183693	p15.2
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AB	AB	AB	AB	NoCall	AB	rs795541	p15.2	AB	AB	AB	BB	AB	BB	rs27116	p15.2
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IV:14	V:7	V:8	III:4	IV:9	MCDR3 AD	dbSNP RS ID	Cytoband	IV:14	V:7	V:8	III:4	IV:9	MCDR3 AD	dbSNP RS ID	Cytoband
AA	AA	AA	AA	AA	AA	rs27108	p15.2	BB	BB	NoCall	NoCall	BB	BB	rs304578	p15.1
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AA	NoCall	AA	AA	AA	AA	rs26185	p15.2	NoCall	AA	AA	AA	AA	AA	rs16903868	p15.1
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NoCall	AA	AA	AA	AA	AA	rs26182	p15.2	AA	AA	AA	AA	NoCall	AA	rs16903889	p15.1
NoCall	NoCall	NoCall	AB	NoCall	AB	rs32531	p15.2	NoCall	NoCall	NoCall	BB	BB	BB	rs680474	p15.1
AA	AA	AA	AA	AA	AA	rs6882373	p15.2	AB	AB	AB	AB	AA	AA	rs581957	p15.1
BB	BB	BB	BB	NoCall	BB	rs26111	p15.2	BB	AB	BB	AB	AB	BB	rs4144522	p15.1
AA	AA	NoCall	AA	AA	AA	rs27479	p15.2	AB	AB	AA	AB	NoCall	AA	rs6889573	p15.1
NoCall	NoCall	NoCall	NoCall	NoCall	AB	rs152433	p15.2	NoCall	BB	NoCall	BB	NoCall	BB	rs9312897	p15.1
AA	AA	AA	AB	AA	AA	rs9942367	p15.2	NoCall	AA	AA	AA	AB	AA	rs7704791	p15.1
NoCall	NoCall	BB	BB	NoCall	BB	rs32812	p15.2	NoCall	BB	BB	BB	BB	BB	rs4702089	p15.1
AA	AB	AA	AA	AA	AA	rs26217	p15.2	BB	BB	BB	BB	AB	BB	rs6860057	p15.1
BB	BB	BB	BB	BB	BB	rs16903541	p15.2	NoCall	NoCall	NoCall	NoCall	NoCall	AB	rs17602097	p15.1
AB	AB	BB	AB	AB	BB	rs152639	p15.2	AB	NoCall	NoCall	AB	NoCall	AB	rs12152734	p15.1
AB	AA	AA	AA	NoCall	AA	rs152624	p15.2	AB	AB	BB	AB	BB	BB	rs7704590	p15.1
NoCall	BB	BB	BB	BB	BB	rs17248579	p15.2	NoCall	AB	BB	AB	AB	BB	rs4374757	p15.1
BB	BB	NoCall	NoCall	NoCall	BB	rs16903568	p15.2	NoCall	AB	BB	AB	BB	BB	rs16867615	p15.1
NoCall	AA	AA	AA	AA	AA	rs456180	p15.2	AB	AB	AA	AB	AA	AA	rs16867617	p15.1
NoCall	AA	AA	AA	AA	AA	rs95492	p15.2	AA	NoCall	AA	AA	AA	AA	rs17647886	p15.1
BB	BB	BB	BB	BB	BB	rs152785	p15.2	AB	AB	AA	AB	NoCall	AA	rs4289558	p15.1
AB	AA	AA	AA	AA	AA	rs31920	p15.2	BB	BB	BB	BB	BB	BB	rs4702104	p15.1
AB	BB	BB	BB	BB	BB	rs31930	p15.2	AA	AA	AA	AA	AB	AA	rs7714352	p15.1
AB	NoCall	BB	BB	BB	BB	rs25992	p15.2	NoCall	AA	AA	AA	AA	AA	rs9637832	p15.1
NoCall	NoCall	NoCall	AA	AA	AA	rs25988	p15.2	NoCall	AA	NoCall	NoCall	AA	AA	rs16867713	p15.1
AB	NoCall	AA	AA	NoCall	AA	rs25987	p15.2	BB	AB	AB	AB	NoCall	BB	rs399259	p15.1
AA	AA	AA	AB	AA	AA	rs149391	p15.2	AA	AA	AA	AA	AA	AA	rs10520826	p15.1
AA	AA	AA	AB	NoCall	AA	rs153926	p15.2	AA	AA	AA	AA	AA	AA	rs10474718	p15.1
AA	AA	AA	AB	AA	AA	rs697568	p15.2	AA	AB	AB	AB	AA	AA	rs7726523	p15.1
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AA	AA	AA	AA	AA	AA	rs10475028	p15.2	AB	AB	AB	AB	AB	AB	rs1393268	p15.1
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AA	NoCall	AB	AB	AA	AA	rs27353	p15.2	NoCall	NoCall	NoCall	NoCall	NoCall	AB	rs6554910	p15.1
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AA	AA	AB	AA	AA	AA	rs10474705	p15.2	AA	NoCall	AA	AA	AA	AA	rs2121182	p15.1
AA	AB	AA	AB	AA	AA	rs1550826	p15.2	BB	BB	BB	BB	BB	BB	rs344701	p15.1
AA	AA	AB	AA	AA	AA	rs1004672	p15.2	AA	AA	AA	AA	AA	AA	rs3910973	p15.1
BB	NoCall	NoCall	BB	BB	BB	rs6893519	p15.2	BB	BB	BB	BB	BB	BB	rs344735	p15.1
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AA	AA	AA	NoCall	AA	AA	rs2250599	p15.2	AA	AA	AA	AA	AA	AA	rs171098	p15.1
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BB	BB	BB	BB	NoCall	BB	rs16903723	p15.2	AA	AA	NoCall	NoCall	NoCall	AA	rs1017492	p15.1
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NoCall	AB	AA	AA	NoCall	AA	rs835149	p15.2	BB	BB	BB	NoCall	BB	BB	rs1992363	p15.1
NoCall	AB	NoCall	NoCall	NoCall	AB	rs835148	p15.2	NoCall	BB	NoCall	NoCall	AB	BB	rs16868296	p15.1
BB	BB	BB	BB	BB	BB	rs2592019	p15.2	AB	AB	AA	AB	AA	AA	rs1440811	p15.1
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AA	AA	NoCall	AA	AB	AA	rs835101	p15.2	BB	BB	BB	BB	NoCall	BB	rs7701965	p15.1
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BB	NoCall	BB	BB	AB	BB	rs12521797	p15.2	BB	BB	BB	BB	BB	BB	rs1579718	p15.1
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NoCall	AA	AA	AA	AA	AA	rs6864877	p15.2	NoCall	BB	NoCall	BB	BB	BB	rs16868520	p15.1
NoCall	NoCall	NoCall	AA	AA	AA	rs10513195	p15.2	BB	BB	AB	BB	NoCall	BB	rs17649710	p15.1
AB	NoCall	AB	BB	AB	BB	rs30541	p15.2	NoCall	NoCall	NoCall	NoCall	NoCall	AB	rs6880065	p15.1
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AA	AA	AB	AB	AB	AA	rs251550	p15.1	BB	BB	BB	NoCall	NoCall	BB	rs335945	p15.1
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AB	BB	AB	AB	AB	BB	rs10067799	p15.1	BB	BB	BB	AB	NoCall	BB	rs26377	p15.1
AB	NoCall	AB	AB	AB	AB	rs173556	p15.1	BB	BB	AB	AB	NoCall	BB	rs149511	p15.1
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AA	AA	AA	NoCall	NoCall	AA	rs16903846	p15.1	AB	AA	AB	AB	AA	AA	rs1867723	p15.1
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AB	NoCall	AB	AB	NoCall	AB	rs897817	p15.1	BB	NoCall	NoCall	NoCall	NoCall	BB	rs334470	p15.1
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AA	AA	AA	AA	AA	AA	rs10036920	p15.1	BB	BB	BB	BB	BB	BB	rs16868716	p15.1
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NoCall	NoCall	NoCall	BB	BB	BB	rs9312882	p15.1	AB	AB	BB	BB	AB	BB	rs2592031	p15.1
AB	BB	AB	AB	AB	BB	rs303345	p15.1	BB	BB	AB	AB	BB	BB	rs6886993	p15.1
AB	AA	AB	AB	AB	AA	rs1447277	p15.1	AA	AA	AA	AA	AA	AA	rs6887114	p15.1
BB	NoCall	BB	BB	NoCall	BB	rs897816	p15.1	AA	AA	AB	AB	AA	AA	rs6862083	p15.1
NoCall	NoCall	NoCall	BB	NoCall	BB	rs7736609	p15.1	NoCall	NoCall	NoCall	AB	AA	AA	rs10041159	p15.1
NoCall	AA	AA	NoCall	NoCall	AA	rs303354	p15.1	AB	AB	NoCall	NoCall	AB	AB	rs2617421	p15.1
NoCall	NoCall	BB	NoCall	NoCall	BB	rs7719232	p15.1	AB	AB	BB	BB	AB	BB	rs1240649	p15.1
NoCall	AA	NoCall	AA	AA	AA	rs6880299	p15.1	NoCall	AA	AB	NoCall	NoCall	AA	rs2560828	p15.1
AB	AA	AB	AB	AB	AA	rs177360	p15.1	BB	BB	BB	BB	BB	BB	rs17650584	p15.1
AB	AA	AB	AB	AB	AA	rs303355	p15.1	BB	BB	NoCall	BB	NoCall	BB	rs431154	p15.1
AA	AA	AA	AA	AA	AA	rs4701628	p15.1	AA	AA	NoCall	AA	AA	AA	rs403565	p15.1
AA	NoCall	NoCall	NoCall	NoCall	AA	rs9968700	p15.1	AB	AA	AA	AB	AB	AA	rs6887031	p15.1
BB	NoCall	BB	NoCall	NoCall	BB	rs402623	p15.1	BB	AB	BB	BB	AB	BB	rs26607	p15.1
BB	BB	BB	BB	BB	BB	rs11133817	p15.1	AA	NoCall	AA	AA	AB	AA	rs35001	p15.1
BB	BB	NoCall	NoCall	BB	BB	rs304578	p15.1	BB	AB	AB	AB	BB	BB	rs4482904	p15.1

IV:14	V:7	V:8	III:4	IV:9	MCDR3 AD	dbSNP RS ID	Cytoband
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AB	BB	AB	BB	BB	BB	rs2560837	p15.1
AB	BB	NoCall	BB	BB	BB	rs2561234	p15.1
AB	BB	NoCall	BB	BB	BB	rs257159	p15.1
AA	AB	AA	AB	AA	AA	rs27553	p15.1
BB	AB	BB	BB	BB	BB	rs4702164	p15.1
AB	BB	AB	BB	NoCall	BB	rs35580	p15.1
BB	AB	BB	AB	NoCall	BB	rs2303703	p15.1
AA	AA	AA	AA	NoCall	AA	rs256934	p15.1
NoCall	BB	BB	BB	NoCall	BB	rs181940	p15.1
AA	AA	AA	NoCall	AA	AA	rs16869070	p15.1
NoCall	AB	BB	AB	AB	BB	rs4702171	p15.1
BB	AB	NoCall	AB	BB	BB	rs890910	p15.1
NoCall	BB	BB	AB	BB	BB	rs11133860	p15.1
BB	AB	BB	BB	BB	BB	rs6877202	p15.1
NoCall	AB	AA	NoCall	AA	AA	rs250350	p15.1
NoCall	NoCall	NoCall	NoCall	NoCall	AB	rs7737987	p15.1
BB	AB	BB	AB	BB	BB	rs496033	p15.1
AB	AB	AB	BB	AB	BB	rs31505	p15.1
NoCall	NoCall	NoCall	NoCall	AB	AB	rs10434655	p15.1
BB	BB	NoCall	NoCall	NoCall	BB	rs17707882	p15.1
NoCall	AA	NoCall	AA	AA	AA	rs253314	p15.1
AA	AA	AA	AA	AA	AA	rs253313	p15.1
AA	AA	AA	AA	AA	AA	rs253312	p15.1
BB	BB	BB	NoCall	NoCall	BB	rs253311	p15.1
AA	AA	AA	AA	AA	AA	rs17707924	p15.1
AA	NoCall	AA	AA	NoCall	AA	rs39896	p15.1
BB	BB	BB	BB	BB	BB	rs13178744	p15.1
BB	NoCall	BB	BB	BB	BB	rs31452	p15.1
AB	AA	AA	AA	AA	AA	rs10061139	p15.1
AB	AA	AA	NoCall	AA	AA	rs2402335	p15.1
BB	BB	BB	BB	BB	BB	rs7734901	p15.1
AB	BB	BB	BB	AB	BB	rs860537	p15.1
BB	BB	BB	BB	BB	BB	rs4129967	p15.1
AA	AA	AA	AA	AA	AA	rs11953330	p15.1
BB	BB	BB	BB	BB	BB	rs11959796	p15.1
AA	AA	AA	AA	AA	AA	rs6866220	p15.1
AA	AA	AA	AA	AA	AA	rs11949558	p15.1
NoCall	BB	BB	AB	NoCall	BB	rs11133874	p15.1
AB	AA	AA	AB	AA	AA	rs6884918	p15.1
AB	BB	BB	AB	BB	BB	rs4072898	p15.1
AA	NoCall	NoCall	NoCall	NoCall	AA	rs2086607	p15.1
AA	AA	AA	AA	AA	AA	rs4327598	p15.1
BB	BB	BB	BB	BB	BB	rs17542943	p15.1
BB	NoCall	BB	BB	BB	BB	rs7717444	p15.1
AA	AA	AA	NoCall	NoCall	AA	rs4235557	p15.1
NoCall	NoCall	BB	NoCall	NoCall	BB	rs4702197	p15.1
NoCall	AA	NoCall	AA	AA	AA	rs4583886	p15.1
AA	NoCall	AA	AA	NoCall	AA	rs4620026	p15.1
AB	NoCall	NoCall	AB	NoCall	AB	rs12655126	p15.1
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AA	AB	AA	AA	AB	AA	rs167213	p15.1
NoCall	AB	AB	BB	AB	BB	rs13175997	p15.1
BB	BB	BB	BB	BB	BB	rs642276	p15.1
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AA	AA	AA	AA	AA	AA	rs2896248	p15.1
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BB	NoCall	NoCall	AB	BB	BB	rs297194	p15.1
AA	AA	AA	AA	AA	AA	rs2956560	p15.1
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BB	AB	BB	BB	BB	BB	rs298542	p15.1
NoCall	AB	AA	AB	NoCall	AA	rs953431	p15.1
BB	BB	BB	BB	BB	BB	rs298592	p15.1
NoCall	NoCall	NoCall	AA	NoCall	AA	rs167237	p15.1
AA	NoCall	AA	AA	AA	AA	rs298572	p15.1
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AA	AA	AA	AB	AA	AA	rs2036851	p15.1
NoCall	BB	BB	BB	BB	BB		p15.1
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AB	BB	NoCall	AB	BB	BB	rs159606	p15.1
BB	BB	NoCall	BB	BB	BB	rs2459811	p15.1
NoCall	BB	AB	AB	BB	BB	rs2008011	p15.1
AB	AA	AB	AB	AA	AA	rs867356	p15.1
AB	AA	AB	AA	NoCall	AA	rs870893	p15.1
AB	AB	AB	BB	AB	BB	rs3733746	p15.1
NoCall	AB	NoCall	BB	NoCall	BB	rs4318775	p15.1
AA	AB	AA	AA	AA	AA	rs6894826	p15.1
AB	AB	AB	AB	NoCall	AB	rs7718603	p15.1
AA	AA	NoCall	AA	NoCall	AA	rs6451988	p15.1
BB	AB	BB	BB	BB	BB	rs1514846	p15.1
AA	AB	NoCall	AA	NoCall	AA	rs13190307	p15.1
AA	NoCall	NoCall	AB	AA	AA	rs6868901	p15.1
BB	BB	BB	AB	BB	BB	rs6863070	p15.1
AA	AA	AA	AB	AA	AA	rs2379266	p15.1
BB	BB	AB	AB	BB	BB	rs9292706	p15.1
BB	BB	BB	NoCall	BB	BB	rs1514867	p15.1
NoCall	BB	AB	BB	BB	BB	rs6451281	p15.1
AA	NoCall	AA	AA	NoCall	AA	rs16887640	p15.1

IV:14	V:7	V:8	III:4	IV:9	MCDR3 AD	dbSNP RS ID	Cytoband
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AB	NoCall	NoCall	AA	AA	AA	rs1398596	p15.1
NoCall	AB	NoCall	NoCall	NoCall	AB	rs7731579	p15.1
AB	AB	AB	BB	BB	BB	rs13188408	p15.1
AA	AA	NoCall	AA	NoCall	AA	rs690799	p15.1
AB	AB	AB	AB	AB	AB	rs569786	p15.1
NoCall	AB	NoCall	AB	NoCall	AB	rs526047	p15.1
BB	NoCall	NoCall	NoCall	NoCall	BB	rs254828	p15.1
AB	AB	AB	AB	AA	AA	rs6867384	p15.1
AB	AB	AB	AB	AA	AA	rs6891956	p15.1
AA	AA	AA	AA	AA	AA	rs17839277	p15.1
AB	AB	AB	AB	BB	BB	rs11952141	p15.1
AA	AA	AA	NoCall	AA	AA	rs1467955	p15.1
AA	AA	AA	AA	AA	AA	rs643091	p15.1
AA	AA	AA	AA	AA	AA	rs2652554	p15.1
BB	AB	AB	AB	AA	Exclude	rs681129	p15.1
AB	NoCall	BB	NoCall	NoCall	BB	rs17660947	p15.1
AB	NoCall	AA	NoCall	NoCall	AA	rs17661079	p15.1
BB	BB	BB	BB	BB	BB	rs17712061	p15.1
AA	NoCall	AA	AB	BB	Exclude	rs2443547	p15.1
AB	AB	AA	AB	NoCall	AA	rs2923765	p15.1
NoCall	NoCall	BB	AB	NoCall	BB	rs315825	p15.1
AB	AB	BB	AB	AA	Exclude	rs2938412	p15.1
AA	AB	AA	AB	BB	Exclude	rs453891	p15.1
NoCall	NoCall	AB	NoCall	NoCall	AB	rs314019	p15.1
AA	AA	AA	AA	AA	AA	rs2962895	p15.1
AA	AA	AB	AB	BB	Exclude	rs2950484	p15.1
BB	NoCall	BB	AB	AA	Exclude	rs10461822	p15.1
NoCall	NoCall	NoCall	NoCall	NoCall	AB	rs2962928	p15.1
AB	NoCall	NoCall	AA	NoCall	AA	rs17630739	p15.1
AA	AA	NoCall	AA	AA	AA	rs1880945	p15.1
BB	BB	AB	AB	AA	Exclude	rs2962902	p15.1
BB	BB	BB	BB	BB	BB	rs1527386	p15.1
NoCall	NoCall	AB	NoCall	NoCall	AB	rs1527387	p15.1
AB	BB	BB	NoCall	BB	BB	rs4866333	p15.1
AB	BB	BB	BB	BB	BB	rs6870006	p14.3
NoCall	BB	BB	BB	BB	BB	rs1527383	p14.3

13.4 Appendix IV

Primers for exons 1 and 6 for failed *NOVA1* screening.

Figure 13.2 Primer positions attempted for amplification of *NOVA1* exons 1 (top) and 6 (bottom).

Table 13.2 Primers for *NOVA1* exons 1 and 6, screening for which failed.

Primers	Name
GAGAAATAATGAGCGAGACCG	1F
AATGGAAATGTGTCGGGGACA	1R
TACATGGTCAACCTCTGGACCGAT	NEW 1R
TCTAGGATAGCAAAGTAAT	6AF
GATCTGTATCCTTGCACCAG	6AR
CTGGTGCAAGGATACAGATC	6BF
ACCACAGTTATGTGTCTA	6BR
TAGACACATAACTGTGGT	6CF
CTGAGACTTGTGACATGC	6CR

13.5 Appendix V

MCDR4 14q 250K genotyping data plus examples from other chromosomes (1, 5 and 16) as examples showing all chromosome data had a similar AD exclusion pattern.

Chromosome 14q11.2:

III:3	IV:4	II:3	III:7	IV:6	IV:7	MCDR4 AD	dbSNP RS ID	Cytoband
AA	AA	NoCall	NoCall	AB	AB	AA	rs4983173	q11.2
NoCall	AA	AA	NoCall	AA	NoCall	AA	rs4562981	q11.2
AB	AB	AB	NoCall	BB	NoCall	BB	rs4558339	q11.2
BB	NoCall	NoCall	AB	AB	AB	BB	rs4473104	q11.2
AB	AB	AB	AB	AB	AB	AB	rs1780909	q11.2
BB	NoCall	BB	BB	BB	BB	BB	rs2318498	q11.2
AA	AA	AA	AA	AA	AA	AA	rs4359352	q11.2
BB	NoCall	BB	NoCall	NoCall	BB	BB	rs17242341	q11.2
AB	NoCall	NoCall	AB	AB	AB	AB	rs1686588	q11.2
AA	NoCall	AA	NoCall	AB	AB	AA	rs6572904	q11.2
AB	NoCall	NoCall	AB	AB	AB	AB	rs3916621	q11.2
NoCall	NoCall	NoCall	NoCall	NoCall	AA	AA	rs1953978	q11.2
NoCall	BB	AB	AB	AA	NoCall	Exclude	rs1953998	q11.2
AB	AB	AB	AB	NoCall	NoCall	AB	rs10484226	q11.2
NoCall	AA	AA	AA	AA	AA	AA	rs1959641	q11.2
AB	AA	AB	AB	BB	BB	Exclude	rs2150324	q11.2
AB	AA	AA	AB	AB	AB	AA	rs2210191	q11.2
BB	BB	BB	BB	BB	BB	BB	rs10143483	q11.2
AB	BB	BB	AB	AB	AB	BB	rs872258	q11.2
BB	BB	BB	BB	BB	BB	BB	rs1188561	q11.2
BB	BB	BB	BB	NoCall	BB	BB	rs1188578	q11.2
NoCall	BB	BB	AB	AB	AB	BB	rs1952808	q11.2
AA	AB	AB	AA	AA	AA	AA	rs12431940	q11.2
BB	AB	AB	NoCall	BB	BB	BB	rs10498265	q11.2
AA	AB	AB	AB	AB	AB	AA	rs1953558	q11.2
AA	AB	AB	AB	NoCall	AB	AA	rs1953559	q11.2
NoCall	AB	BB	BB	BB	BB	BB	rs17277270	q11.2
AB	AA	NoCall	AA	AA	AA	AA	rs11851004	q11.2
AB	BB	NoCall	NoCall	NoCall	BB	BB	rs10139180	q11.2
BB	BB	BB	NoCall	NoCall	AB	BB	rs2318860	q11.2
AA	AA	AA	AB	AB	NoCall	AA	rs12589751	q11.2
BB	NoCall	BB	AB	AA	NoCall	Exclude	rs10134743	q11.2
BB	BB	BB	AB	NoCall	AA	Exclude	rs11623837	q11.2
AA	AA	AB	AA	AB	AB	AA	rs2318864	q11.2
AA	AA	AB	AA	AB	AB	AA	rs10873395	q11.2
AA	NoCall	AB	AA	AA	AA	AA	rs7153417	q11.2
BB	BB	BB	BB	BB	BB	BB	rs3093872	q11.2
AB	BB	BB	BB	BB	NoCall	BB	rs4981158	q11.2
AB	BB	BB	BB	BB	BB	BB	rs7161611	q11.2
BB	AB	AB	BB	BB	BB	BB	rs1713448	q11.2
AA	NoCall	AB	AA	AA	AA	AA	rs1760907	q11.2
AA	NoCall	AA	NoCall	NoCall	AA	AA	rs4982038	q11.2
BB	BB	AB	BB	BB	BB	BB	rs1713417	q11.2
AA	AA	AB	AA	AA	NoCall	AA	rs938892	q11.2
AA	AA	AA	AA	AA	AA	AA	rs11160462	q11.2
BB	BB	NoCall	BB	BB	BB	BB	rs17111211	q11.2
NoCall	NoCall	NoCall	BB	NoCall	NoCall	BB	rs2275009	q11.2
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AB	NoCall	AB	AB	AA	AA	AA	rs1760955	q11.2
AB	AA	AA	AB	AB	AB	AA	rs1953226	q11.2
AB	AB	AB	AB	NoCall	AA	AA	rs12436843	q11.2
BB	NoCall	NoCall	NoCall	NoCall	NoCall	BB	rs12587478	q11.2
NoCall	BB	BB	BB	NoCall	NoCall	BB	rs11160645	q11.2
AA	AA	AA	NoCall	AA	AA	AA	rs17211432	q11.2
NoCall	AB	AA	AA	AA	AA	AA	rs6575879	q11.2
AB	AB	AA	AA	NoCall	AA	AA	rs8010845	q11.2
AB	AB	BB	BB	BB	BB	BB	rs1878705	q11.2
NoCall	AB	AB	AB	AA	AA	AA	rs11160711	q11.2
AB	BB	BB	BB	AB	AB	BB	rs938881	q11.2
BB	AB	AB	AB	AB	AB	BB	rs999692	q11.2
AB	BB	BB	BB	AB	AB	BB	rs883037	q11.2
AA	AA	NoCall	AA	AA	NoCall	AA	rs3136818	q11.2
NoCall	BB	BB	BB	BB	BB	BB	rs1065749	q11.2
AA	AB	AA	AA	AA	AA	AA	rs11850059	q11.2
AA	AB	AA	AA	AA	AA	AA	rs11850069	q11.2
BB	AB	BB	BB	BB	BB	BB	rs17242662	q11.2
NoCall	BB	BB	NoCall	BB	BB	BB	rs12147450	q11.2
AA	AA	AA	AA	AA	AA	AA	rs17308401	q11.2
AA	AA	AA	AA	AA	AA	AA	rs17112431	q11.2
AA	AA	AA	AA	AB	AB	AA	rs12891826	q11.2
AB	AB	AB	BB	AB	AB	BB	rs7161172	q11.2
AA	AA	AA	AA	NoCall	NoCall	AA	rs2840281	q11.2
AB	AB	AB	AA	NoCall	AB	AA	rs11628127	q11.2
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BB	BB	BB	NoCall	BB	BB	BB	rs7159719	q11.2
BB	NoCall	NoCall	BB	BB	BB	BB	rs1243704	q11.2
NoCall	AA	AA	AA	AA	AA	AA	rs7148897	q11.2
BB	BB	NoCall	BB	BB	BB	BB	rs17242692	q11.2
NoCall	NoCall	NoCall	AA	AA	NoCall	AA	rs1243698	q11.2
BB	BB	BB	BB	BB	BB	BB	rs1243648	q11.2
AB	AA	AA	AA	AA	AA	AA	rs6576288	q11.2
AB	BB	BB	BB	BB	BB	BB	rs10151120	q11.2
AA	AB	AB	AA	AA	AA	AA	rs4981260	q11.2
AA	AB	AB	AB	BB	BB	Exclude	rs4981261	q11.2
AA	AB	AB	AB	BB	BB	Exclude	rs4981265	q11.2
AA	AA	AA	AA	AA	AA	AA	rs8018826	q11.2
BB	AB	BB	BB	AB	NoCall	BB	rs11622794	q11.2
BB	AB	NoCall	BB	AB	AB	BB	rs11156622	q11.2
AA	AB	AA	AA	AB	AB	AA	rs2115656	q11.2
BB	AB	BB	BB	AB	AB	BB	rs2319378	q11.2
AB	AB	AA	AA	AB	AB	AA	rs12893510	q11.2
NoCall	AB	AB	BB	BB	BB	BB	rs8003288	q11.2
BB	NoCall	AB	BB	BB	BB	BB	rs7158152	q11.2
AB	AA	AA	AB	AB	AB	AA	rs9322855	q11.2
NoCall	AA	AA	NoCall	NoCall	NoCall	AA	rs1010461	q11.2
AB	AB	BB	AB	AB	AB	BB	rs5019558	q11.2
AB	BB	BB	BB	BB	BB	BB	rs17114671	q11.2
AB	NoCall	NoCall	BB	BB	BB	BB	rs8008440	q11.2
NoCall	AA	AA	AA	AA	AA	AA	rs11156631	q11.2

III:3	IV:4	II:3	III:7	IV:6	IV:7	MCDR4 AD	dbSNP RS ID	Cytoband
NoCall	AA	AA	AA	AA	AA	AA	rs11156631	q11.2
BB	BB	BB	BB	BB	BB	BB	rs1999343	q11.2
NoCall	BB	BB	BB	BB	BB	BB	rs17242790	q11.2
AA	AB	AA	AA	AA	AA	AA	rs17211649	q11.2
AB	AB	AA	AB	BB	BB	Exclude	rs4982343	q11.2
NoCall	AA	AA	NoCall	NoCall	AA	AA	rs8004317	q11.2
AB	AB	BB	AB	AA	AA	Exclude	rs4982347	q11.2
AA	AB	BB	AB	AB	AB	Exclude	rs2319444	q11.2
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NoCall	BB	BB	BB	BB	BB	BB	rs17308541	q11.2
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NoCall	AB	AA	NoCall	NoCall	AB	AA	rs8004417	q11.2
BB	BB	BB	NoCall	BB	BB	BB	rs11848221	q11.2
NoCall	AB	NoCall	AB	AB	AB	AB	rs11624594	q11.2
AA	AA	NoCall	AA	AA	AA	AA	rs12895364	q11.2
BB	BB	BB	BB	BB	BB	BB	rs10150727	q11.2
BB	AB	AA	AB	AB	AB	Exclude	rs762038	q11.2
NoCall	AB	AB	BB	BB	BB	BB	rs2009021	q11.2
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AB	BB	AA	AA	AA	AA	Exclude	rs1045922	q11.2
AB	AA	NoCall	AA	NoCall	AA	AA	rs8013362	q11.2
BB	BB	AB	AA	AB	AB	Exclude	rs8009569	q11.2
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NoCall	AA	AA	AA	AA	AA	AA	rs17096637	q11.2
AA	NoCall	AA	AA	AA	AA	AA	rs17096638	q11.2
NoCall	BB	BB	BB	BB	BB	BB	rs1543489	q11.2
NoCall	BB	BB	BB	NoCall	BB	BB	rs8017622	q11.2
AA	AA	AA	AA	AA	AA	AA	rs8018145	q11.2
AA	AA	NoCall	AA	NoCall	AA	AA	rs17254457	q11.2
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AA	AA	AA	AA	NoCall	AA	AA	rs6571504	q11.2
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AA	AB	AB	AB	AB	AB	AA	rs1958399	q11.2
BB	BB	AB	BB	BB	BB	BB	rs1889774	q11.2
BB	AB	AB	BB	BB	BB	BB	rs2771335	q11.2
AB	AB	BB	BB	BB	BB	BB	rs2771354	q11.2
AA	AA	BB	AB	NoCall	AB	Exclude	rs1243446	q11.2
AB	AA	NoCall	AB	AB	AB	AA	rs1243450	q11.2
AB	BB	AA	AA	AA	AA	Exclude	rs1243459	q11.2
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BB	AB	AB	AB	AB	NoCall	BB	rs7149011	q11.2
BB	AB	BB	AB	AB	AB	BB	rs745696	q11.2
AA	AA	AA	AA	AA	NoCall	AA	rs8023143	q11.2
AB	AA	AB	AB	AB	AB	AA	rs4982419	q11.2
AB	BB	AB	NoCall	BB	BB	BB	rs4058351	q11.2
AB	BB	AB	BB	BB	BB	BB	rs7148898	q11.2
NoCall	BB	AB	BB	BB	BB	BB	rs8017588	q11.2
AA	AA	BB	AB	AA	AA	Exclude	rs10140795	q11.2
AA	AA	BB	AB	AB	AB	Exclude	rs2319685	q11.2
BB	AB	AA	AB	AB	AB	Exclude	rs10483251	q11.2
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AA	AA	AA	AA	AA	AA	AA	rs17102539	q11.2
BB	BB	AB	BB	AB	AB	BB	rs7495	q11.2
BB	BB	AB	BB	NoCall	AB	BB	rs8009894	q11.2
AA	AA	AA	AA	NoCall	NoCall	AA	rs4982437	q11.2
NoCall	BB	BB	BB	NoCall	NoCall	BB	rs2065093	q11.2
AA	AA	AB	AA	AB	AB	AA	rs7141230	q11.2
BB	BB	BB	BB	AB	AB	BB	rs17092523	q11.2
BB	BB	BB	BB	BB	BB	BB	rs10145150	q11.2
BB	BB	BB	BB	BB	BB	BB	rs8021748	q11.2
AA	AA	AA	AA	AA	AA	AA	rs7157052	q11.2
NoCall	NoCall	NoCall	NoCall	NoCall	AA	AA	rs2295865	q11.2
AA	AA	AA	AA	AA	AA	AA	rs10872898	q11.2
AA	AA	AA	AA	AA	AA	AA	rs10782384	q11.2
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BB	NoCall	BB	BB	NoCall	BB	BB	rs2146062	q11.2
AA	AA	AA	AA	AA	AA	AA	rs10132333	q11.2
BB	BB	BB	BB	BB	BB	BB	rs7152833	q11.2
AA	AA	AA	AA	AA	AA	AA	rs17792659	q11.2
AA	AA	BB	BB	AB	AB	Exclude	rs10135215	q11.2
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AA	NoCall	AA	AA	AA	NoCall	AA	rs8016621	q11.2
AB	NoCall	AA	AA	AB	AB	AA	rs10129436	q11.2
AB	AB	AA	AB	BB	BB	Exclude	rs11626693	q11.2
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NoCall	BB	AB	AA	AB	AB	Exclude	rs8011308	q11.2
AB	AB	AB	AA	AA	AA	AA	rs2049787	q11.2
BB	NoCall	BB	AB	AB	AB	BB	rs8008166	q11.2
NoCall	AA	AB	NoCall	AB	AB	AA	rs12897485	q11.2
AB	NoCall	AB	NoCall	AB	NoCall	AB	rs10141478	q11.2
BB	BB	BB	BB	BB	BB	BB	rs17109245	q11.2
AB	BB	AB	AA	AB	AB	Exclude	rs9323020	q11.2
AA	AA	AA	AA	AA	AA	AA	rs12436315	q11.2
AB	AA	AB	BB	AB	AB	Exclude	rs28575550	q11.2
AA	AA	AA	AB	AB	AB	AA	rs3817625	q11.2
AB	AB	AB	AB	NoCall	AB	AB	rs10483257	q11.2
NoCall	NoCall	AA	AA	NoCall	AA	AA	rs11846985	q11.2
AB	AB	AB	AA	AA	AA	AA	rs1882699	q11.2
AB	AB	AA	AA	NoCall	AA	AA	rs2254896	q11.2
BB	AB	AA	AA	AB	AB	Exclude	rs11844749	q11.2
AB	AB	AA	AA	AA	AA	AA	rs12888589	q11.2
BB	BB	BB	BB	BB	BB	BB	rs17182830	q11.2

III:3	IV:4	II:3	III:7	IV:6	IV:7	MCDR4 AD	dbSNP RS ID	Cytoband	III:3	IV:4	II:3	III:7	IV:6	IV:7	MCDR4 AD	dbSNP RS ID	Cytoband
BB	BB	BB	BB	BB	BB	BB	rs17182830	q11.2	AA	AA	AA	AB	BB	BB	Exclude	rs1263657	q11.2
AB	AB	BB	BB	BB	BB	BB	rs12880912	q11.2	AB	BB	BB	BB	BB	BB	BB	rs17461	q11.2
AB	AB	BB	NoCall	BB	BB	BB	rs2272545	q11.2	BB	AB	NoCall	NoCall	NoCall	NoCall	BB	rs6572540	q11.2
AB	AB	BB	BB	BB	BB	BB	rs2272546	q11.2	BB	AB	AB	AB	BB	BB	BB	rs4982640	q11.2
NoCall	NoCall	BB	BB	BB	BB	BB	rs2017238	q11.2	BB	AB	AA	AA	AA	AA	Exclude	rs4982641	q11.2
AB	NoCall	BB	AB	AB	AB	BB	rs6572141	q11.2	AA	AA	NoCall	AA	NoCall	NoCall	AA	rs5742848	q11.2
AB	BB	AB	BB	AB	AB	BB	rs2293718	q11.2	BB	AB	AB	AB	BB	BB	BB	rs5742847	q11.2
NoCall	BB	AB	BB	AB	NoCall	BB	rs994826	q11.2	AA	AA	AA	NoCall	AA	AA	AA	rs17119929	q11.2
BB	NoCall	NoCall	BB	AB	NoCall	BB	rs994827	q11.2	AA	AA	AA	AA	AA	AA	AA	rs5742808	q11.2
NoCall	BB	NoCall	NoCall	BB	NoCall	BB	rs2204940	q11.2	BB	BB	BB	BB	BB	BB	BB	rs5742805	q11.2
NoCall	BB	BB	BB	BB	BB	BB	rs2103414	q11.2	AA	AA	AA	AA	AA	AA	AA	rs5742794	q11.2
AB	AA	AB	AA	AB	AB	AA	rs11851092	q11.2	AA	AB	AB	AB	AB	AB	AA	rs8011143	q11.2
NoCall	AA	AB	NoCall	AB	AB	AA	rs8015446	q11.2	BB	AB	AA	AA	AB	AB	Exclude	rs2225215	q11.2
NoCall	AA	AA	NoCall	AA	NoCall	AA	rs8003815	q11.2	AA	AB	BB	AB	AA	AA	Exclude	rs970348	q11.2
AB	AA	AB	AA	AB	AB	AA	rs8009355	q11.2	NoCall	BB	BB	BB	AB	AB	BB	rs1681587	q11.2
BB	AB	AB	AB	BB	BB	BB	rs8014496	q11.2	NoCall	BB	BB	BB	AB	NoCall	BB	rs922955	q11.2
NoCall	BB	NoCall	NoCall	NoCall	BB	BB	rs7159964	q11.2	AB	BB	BB	BB	BB	BB	BB	rs1681595	q11.2
BB	AB	AB	AB	BB	BB	BB	rs1997535	q11.2	AB	AA	AA	AA	AA	AA	AA	rs1753429	q11.2
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AA	AA	AA	AA	AA	NoCall	AA	rs11846714	q11.2	AB	BB	AB	BB	BB	BB	BB	rs1753430	q11.2
AB	BB	NoCall	NoCall	NoCall	NoCall	BB	rs12586624	q11.2	NoCall	BB	BB	BB	BB	BB	BB	rs3829405	q11.2
NoCall	AA	AA	NoCall	AB	AB	AA	rs12433492	q11.2	AA	NoCall	AB	AA	NoCall	AA	AA	rs10147839	q11.2
AB	NoCall	BB	AB	AB	AB	BB	rs2204950	q11.2	AB	AA	AB	NoCall	AA	AA	AA	rs12589700	q11.2
AB	NoCall	NoCall	AB	AB	AB	AB	rs17255175	q11.2	AB	AB	AB	AA	AB	AB	AA	rs9323178	q11.2
NoCall	BB	NoCall	BB	BB	BB	BB	rs932230	q11.2	AA	AA	AA	AA	AA	NoCall	AA	rs1806772	q11.2
BB	BB	BB	NoCall	NoCall	BB	BB	rs932231	q11.2	AB	AB	NoCall	AA	AB	AB	AA	rs10134801	q11.2
AA	AA	AA	AA	AA	AA	AA	rs17197676	q11.2	NoCall	NoCall	NoCall	NoCall	NoCall	NoCall	AB	rs10147403	q11.2
BB	AB	AB	AB	AA	AA	Exclude	rs4982541	q11.2	AA	NoCall	AA	AA	AA	AA	AA	rs17121539	q11.2
AA	NoCall	NoCall	NoCall	NoCall	NoCall	AA	rs10146749	q11.2	NoCall	AA	AA	AA	NoCall	AA	AA	rs4246996	q11.2
BB	BB	AB	AB	BB	BB	BB	rs3811321	q11.2	AA	AA	AA	AA	AA	AA	AA	rs1242734	q11.2
BB	BB	BB	BB	BB	BB	BB	rs7151120	q11.2	BB	BB	BB	BB	BB	BB	BB	rs7148652	q11.2
NoCall	AA	AA	AA	AA	AA	AA	rs12588446	q11.2	BB	AB	BB	NoCall	AB	AB	BB	rs1242744	q11.2
AB	NoCall	AB	AB	NoCall	AA	AA	rs732073	q11.2	BB	AB	AB	BB	AB	AB	BB	rs2877489	q11.2
AB	NoCall	NoCall	AB	NoCall	NoCall	AB	rs10130648	q11.2	AA	AA	NoCall	NoCall	AA	NoCall	AA	rs8786	q11.2
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AA	AB	AA	AB	BB	BB	Exclude	rs3811306	q11.2	NoCall	NoCall	NoCall	NoCall	NoCall	NoCall	AB	rs11568430	q11.2
BB	BB	BB	BB	BB	BB	BB	rs17114015	q11.2	BB	AB	AB	AA	AA	AA	Exclude	rs12433985	q11.2
AB	AB	AA	AB	BB	BB	Exclude	rs6572248	q11.2	NoCall	AB	AB	AB	NoCall	NoCall	AB	rs6572754	q11.2
AA	NoCall	AA	AA	NoCall	NoCall	AA	rs17183131	q11.2	AB	AB	AB	AB	AA	AA	AA	rs8006783	q11.2
AB	NoCall	NoCall	NoCall	NoCall	NoCall	AB	rs7144892	q11.2	BB	BB	BB	BB	BB	BB	BB	rs8006959	q11.2
AA	AA	AA	AB	BB	BB	Exclude	rs17197802	q11.2	BB	BB	BB	BB	BB	BB	BB	rs8005577	q11.2
AA	AA	AA	AA	AA	AA	AA	rs2178785	q11.2	AB	NoCall	BB	NoCall	NoCall	NoCall	BB	rs3829407	q11.2
AB	AB	NoCall	BB	AB	AB	BB	rs1474476	q11.2	AB	AA	NoCall	AB	NoCall	BB	Exclude	rs7157654	q11.2
AA	AA	AA	AA	AA	AA	AA	rs11157435	q11.2	AB	BB	BB	AB	AA	NoCall	Exclude	rs12432242	q11.2
BB	BB	BB	BB	NoCall	BB	BB	rs11157436	q11.2	NoCall	BB	BB	NoCall	AA	AA	Exclude	rs2281678	q11.2
AA	AA	NoCall	AA	AA	AA	AA	rs11157438	q11.2	BB	BB	AB	AB	BB	BB	BB	rs3751488	q11.2
BB	AB	NoCall	BB	BB	BB	BB	rs10139215	q11.2	AA	AA	AB	AB	AB	AB	AA	rs4982689	q11.2
NoCall	AB	NoCall	NoCall	NoCall	NoCall	AB	rs10139447	q11.2	AB	BB	AB	AB	BB	BB	BB	rs10132585	q11.2
NoCall	AB	NoCall	NoCall	BB	BB	BB	rs10142123	q11.2	AA	AA	AA	AA	AA	NoCall	AA	rs4981444	q11.2
NoCall	AB	NoCall	NoCall	AA	NoCall	AA	rs17093133	q11.2	AA	AA	AA	AA	AA	NoCall	AA	rs4982699	q11.2
BB	BB	NoCall	BB	BB	BB	BB	rs17255510	q11.2	BB	BB	BB	BB	BB	BB	BB	rs17124097	q11.2
AA	AB	AB	NoCall	NoCall	NoCall	AA	rs980775	q11.2	AA	AA	AA	AA	AA	AA	AA	rs1242631	q11.2
AA	AA	AB	AA	AA	AA	AA	rs7153175	q11.2	AA	AB	AB	NoCall	AA	AA	AA	rs2295680	q11.2
AB	AB	AA	AA	NoCall	AA	AA	rs17115728	q11.2	BB	AB	AB	NoCall	BB	BB	BB	rs4981449	q11.2
AB	AA	NoCall	BB	BB	BB	Exclude	rs7149646	q11.2	NoCall	AB	AB	AB	BB	BB	BB	rs1956880	q11.2
NoCall	NoCall	NoCall	NoCall	NoCall	NoCall	AB	rs17115801	q11.2	BB	AB	AB	AB	BB	NoCall	BB	rs4982709	q11.2
AA	AA	NoCall	AB	BB	BB	Exclude	rs8011203	q11.2	AA	AA	AA	AA	AA	AA	AA	rs8022177	q11.2
AB	AB	AB	AA	AA	AA	AA	rs8016619	q11.2	AA	AA	AA	AA	AA	AA	AA	rs3794456	q11.2
BB	BB	BB	BB	BB	BB	BB	rs17183456	q11.2	NoCall	NoCall	BB	BB	BB	BB	BB	rs717984	q11.2
NoCall	NoCall	NoCall	BB	BB	BB	BB	rs17116101	q11.2	AA	AA	AA	AA	AA	AA	AA	rs10498279	q11.2
BB	AB	BB	BB	BB	BB	BB	rs4267225	q11.2	AA	AA	AA	AA	AA	AA	AA	rs9972278	q11.2
AB	BB	AB	AB	BB	BB	BB	rs10483272	q11.2	AA	AA	NoCall	NoCall	NoCall	NoCall	AA	rs17126608	q11.2
NoCall	NoCall	NoCall	NoCall	NoCall	NoCall	AB	rs11157508	q11.2	AA	AA	AA	NoCall	NoCall	AA	AA	rs2236136	q11.2
NoCall	AB	NoCall	AB	AB	AB	AB	rs4982625	q11.2	AB	AA	AB	NoCall	NoCall	AB	AA	rs4982729	q11.2
AB	NoCall	AA	AB	BB	BB	Exclude	rs9919897	q11.2	NoCall	AA	AA	AA	AA	AA	AA	rs2236135	q11.2
AA	AB	AA	AA	AA	AA	AA	rs12588964	q11.2	BB	BB	NoCall	NoCall	BB	BB	BB	rs17127069	q11.2
AB	AB	NoCall	AB	AB	AB	AB	rs9919888	q11.2	AA	AA	AA	AA	AA	AA	AA	rs10150355	q11.2
NoCall	NoCall	BB	NoCall	AB	NoCall	BB	rs10142543	q11.2	AB	BB	AA	AB	AB	AB	Exclude	rs2239627	q11.2
NoCall	AA	NoCall	AB	NoCall	AB	AA	rs8011618	q11.2	AB	NoCall	AA	AB	AB	AB	AA	rs10143650	q11.2
BB	AB	BB	BB	BB	BB	BB	rs12891368	q11.2	AA	AA	AA	AA	AB	AB	AA	rs17183905	q11.2
AB	NoCall	NoCall	NoCall	NoCall	NoCall	AB	rs10149541	q11.2	BB	AB	BB	NoCall	BB	BB	BB	rs1955560	q11.2
NoCall	AB	AB	AB	NoCall	NoCall	AB	rs8012481	q11.2	BB	BB	AB	AB	BB	BB	BB	rs977871	q11.2
AA	AB	AB	AB	AB	NoCall	AA	rs6572449	q11.2	BB	BB	AB	AB	BB	BB	BB	rs1055061	q11.2
NoCall	AB	AB	AB	AB	AB	AB	rs10873018	q11.2	AA	AA	AA	AA	AA	AA	AA	rs10149339	q11.2
NoCall	AB	NoCall	AA	AA	AA	AA	rs10483273	q11.2	NoCall	NoCall	BB	NoCall	BB	NoCall	BB	rs13244	q11.2
NoCall	AB	AB	AB	AB	AB	AB	rs2204985	q11.2	AA	AA	AA	NoCall	AB	AB	AA	rs17090828	q11.2
AA	AA	AB	AB	AB	AB	AA	rs11997	q11.2	BB	BB	BB	AB	AB	AB	BB	rs3759611	q11.2
BB	NoCall	AB	AB	AB	AB	BB	rs10483277	q11.2	AA	AA	AA	AB	AB	AB	AA	rs8017550	q11.2
BB	BB	AB	AB	AB	AB	BB	rs2293732	q11.2	AA	AA	NoCall	NoCall	AA	AA	AA	rs11465510	q11.2
AB	AB	NoCall	NoCall	NoCall	BB	BB	rs1076861	q11.2	NoCall	BB	NoCall	NoCall	BB	NoCall	BB	rs11465515	q11.2
AB	NoCall	AA	NoCall	NoCall	AA	AA	rs2220119	q11.2	AA	AB	NoCall	BB	NoCall	BB	Exclude	rs433673	q11.2
AA	AA	AA	AA	AA	AA	AA	rs10483278	q11.2	BB	BB	BB	BB	BB	BB	BB	rs17091352	q11.2
BB	BB	BB	BB	BB	BB	BB	rs909077	q11.2	AA	AA	NoCall	AA	NoCall	NoCall	AA	rs17091453	q11.2
NoCall	BB	NoCall	BB	NoCall	NoCall	BB	rs226988	q11.2	NoCall	AA	AA	AA	AA	AA	AA	rs17091555	q11.2
NoCall	AA	AB	NoCall	AB	AB	AA	rs1872160	q11.2	AA	AB	AB	NoCall	AA	AA	AA	rs12894524	q11.2
NoCall	NoCall	NoCall	NoCall	NoCall	NoCall	AB	rs17118980	q11.2	AA	AB	BB	AB	AB	AB	Exclude	rs2239578	q11.2
AA	AA	AA	AA	AA	AA	AA	rs226997	q11.2	BB	BB	AB	NoCall	BB	BB	BB	rs8005199	q11.2
AA	NoCall	AB	AB	AB	AB	AA	rs1483973	q11.2	AA	AA	AA	AA	AA	AA	AA	rs8014874	q11.2
AB	NoCall	NoCall	AB	AB	AB	AB	rs1483974	q11.2	AA	AA	AA	NoCall	AA	AA	AA	rs2295705	q11.2
BB	BB	BB	BB	NoCall	BB	BB	rs227009	q11.2	AA	NoCall	AA	AA	NoCall	AA	AA	rs961249	q11.2
AB	AB	AB	AB	AB	AB	AB	rs12588667	q11.2	AA	AA	AA	AA	AA	AA	AA	rs17093091	q11.2
NoCall	BB	NoCall	NoCall	NoCall	NoCall	BB	rs227024	q11.2	AA	NoCall	AA	AA	NoCall	NoCall	AA	rs17199205	q11.2
AB	NoCall	AB	NoCall	AB	AB	AB	rs179106	q11.2	BB	BB	BB	BB	BB	BB	BB	rs1555415	q11.2
BB	BB	NoCall	BB	BB	BB	BB	rs11157651	q11.2	BB	BB	BB	BB	BB	BB	BB	rs11852066	q11.2
AA	AA	AB	AB	AB	AB	AA	rs8016421	q11.2	AA	AA	AA	AA	AA	AA	AA	rs223110	q11.2
AB	AB	NoCall	AA	NoCall	NoCall	AA	rs1154155	q11.2	AA	AA	AB	AA	AA	AA	AA	rs222670	q11.2
AB	AB	BB	NoCall	NoCall	BB	BB	rs1154156	q11.2	BB	BB	BB	BB	BB	NoCall	BB	rs17256392	q11.2
AB	AB	BB	BB	BB	BB	BB	rs1263646	q11.2	BB	BB	BB	BB	BB	NoCall	BB	rs223123	q11.2
AB	AB	NoCall	NoCall	NoCall	AA	AA	rs1263647	q11.2	BB	BB	NoCall	BB	NoCall	NoCall	BB	rs3783437	q11.2
AA	AA	NoCall	AB	BB	BB	Exclude	rs1263655	q11.2	AA	AB	AB	AB	AA	AA	AA	rs7145877	q11.2
AA	AA	AA	AB	BB	BB	Exclude	rs1263657	q11.2	AA	AA	NoCall	AA	NoCall	NoCall	AA	rs222681	q11.2

III:3	IV:4	II:3	III:7	IV:6	IV:7	MCDR4 AD	dbSNP RS ID	Cytoband
AA	AA	NoCall	AA	NoCall	NoCall	AA	rs222681	q11.2
BB	BB	BB	AB	AB	AB	BB	rs12897422	q11.2
BB	BB	BB	BB	BB	NoCall	BB	rs12100622	q11.2
NoCall	AA	NoCall	AA	AA	AA	AA	rs222703	q11.2
AA	AB	BB	AB	AB	AB	Exclude	rs222725	q11.2
NoCall	AB	BB	AB	AB	AB	BB	rs222727	q11.2
BB	AB	AA	AB	AB	AB	Exclude	rs222728	q11.2
BB	BB	BB	NoCall	NoCall	BB	BB	rs10136190	q11.2
AA	AA	AA	AA	AA	AA	AA	rs17095410	q11.2
AA	AB	AA	AA	AA	AA	AA	rs17184233	q11.2
BB	BB	BB	BB	BB	NoCall	BB	rs222609	q11.2
NoCall	NoCall	AA	AA	AA	AA	AA	rs222611	q11.2
AA	AA	AA	AA	AA	AA	AA	rs11158273	q11.2
BB	BB	BB	BB	BB	BB	BB	rs17095582	q11.2
NoCall	NoCall	BB	BB	BB	BB	BB	rs221703	q11.2
BB	BB	NoCall	BB	BB	BB	BB	rs913486	q11.2
BB	BB	BB	BB	BB	BB	BB	rs1885592	q11.2
NoCall	BB	NoCall	BB	BB	NoCall	BB	rs221698	q11.2
NoCall	NoCall	BB	BB	BB	BB	BB	rs1438122	q11.2
NoCall	NoCall	AA	AA	AA	NoCall	AA	rs221696	q11.2
BB	AB	BB	BB	AB	AB	BB	rs2067644	q11.2
NoCall	AA	NoCall	AA	NoCall	NoCall	AA	rs221695	q11.2
BB	BB	BB	BB	NoCall	NoCall	BB	rs221689	q11.2
AA	NoCall	AA	AA	NoCall	AA	AA	rs6573309	q11.2
AA	AB	AA	AA	AA	AA	AA	rs8009273	q11.2
NoCall	AB	AA	AA	AA	AA	AA	rs1952407	q11.2
BB	NoCall	BB	BB	BB	BB	BB	rs11850283	q11.2
BB	AB	BB	BB	AB	AB	BB	rs8007721	q11.2
BB	NoCall	NoCall	NoCall	BB	NoCall	BB	rs12433781	q11.2
AB	NoCall	AB	BB	NoCall	BB	BB	rs4982795	q11.2
NoCall	AA	AA	AA	AA	AA	AA	rs17199380	q11.2
AB	AB	AA	AB	AB	AB	AA	rs9323367	q11.2
AB	AB	NoCall	NoCall	NoCall	NoCall	AB	rs1958305	q11.2
NoCall	NoCall	NoCall	NoCall	NoCall	NoCall	AB	rs17184408	q11.2
NoCall	AA	AA	NoCall	NoCall	NoCall	AA	rs1810792	q11.2
AA	NoCall	AA	NoCall	AA	AA	AA	rs1958299	q11.2
AA	AA	AA	AA	AA	AA	AA	rs2031861	q11.2
NoCall	AA	AA	AA	AA	AA	AA	rs6573420	q11.2
AA	AA	AB	AA	AA	AA	AA	rs1957684	q11.2
BB	BB	BB	BB	BB	BB	BB	rs2208927	q11.2
AA	AA	AA	AA	AA	AA	AA	rs17098897	q11.2
NoCall	NoCall	AA	AA	AA	AA	AA	rs10135546	q11.2
NoCall	NoCall	NoCall	NoCall	NoCall	NoCall	AB	rs11628804	q11.2
AA	AA	AA	AA	AA	AA	AA	rs8020121	q11.2
BB	BB	BB	BB	BB	BB	BB	rs4981490	q11.2
AA	AA	AA	AA	AA	AA	AA	rs10483284	q11.2
AB	NoCall	NoCall	NoCall	NoCall	AA	AA	rs8003491	q11.2
NoCall	BB	BB	BB	BB	BB	BB	rs1535562	q11.2
NoCall	NoCall	NoCall	NoCall	NoCall	NoCall	AB	rs8011236	q11.2
AB	BB	BB	NoCall	BB	BB	BB	rs9805889	q11.2
AB	AA	AA	AA	AA	AA	AA	rs10137990	q11.2
AA	AA	AA	AA	AA	AA	AA	rs10142648	q11.2
AB	AA	AB	AA	NoCall	AB	AA	rs8017358	q11.2
AB	BB	AB	NoCall	AA	AA	Exclude	rs1951634	q12
BB	BB	BB	AB	AB	AB	BB	rs3742499	q12
AB	AA	AB	AA	AB	AB	AA	rs6573565	q12
AB	BB	AB	NoCall	AA	AA	Exclude	rs927494	q12
AB	AA	AB	AA	AB	AB	AA	rs11574503	q12
AB	BB	AB	AB	AA	AA	Exclude	rs3742500	q12
NoCall	AA	AA	AA	AA	AA	AA	rs11844422	q12
AA	AA	AB	AA	AB	AB	AA	rs12892579	q12
BB	AB	BB	AB	AB	AB	BB	rs4568	q12
AA	AA	AA	AA	AA	AA	AA	rs10134537	q12
AA	AA	AB	AA	AB	AB	AA	rs2295306	q12
NoCall	NoCall	AB	NoCall	AB	AB	AB	rs3825586	q12
BB	AB	NoCall	AB	AA	AA	Exclude	rs11158632	q12
AA	AA	AA	AA	NoCall	NoCall	AA	rs4982895	q12
NoCall	NoCall	BB	NoCall	BB	BB	BB	rs1053648	q12
BB	AB	AB	NoCall	AA	AA	Exclude	rs3181384	q12
AA	AB	AB	AB	BB	BB	Exclude	rs4981504	q12
BB	BB	AB	BB	NoCall	AB	BB	rs12891732	q12
AA	NoCall	NoCall	NoCall	NoCall	AA	AA	rs1950502	q12
AA	AA	AB	AA	AB	AB	AA	rs1950501	q12
AA	AB	AB	AB	BB	BB	Exclude	rs8008406	q12
NoCall	AB	AA	AA	AA	AA	AA	rs12433338	q12
BB	BB	AB	BB	AB	AB	BB	rs2295298	q12
BB	AB	AB	AB	NoCall	AA	Exclude	rs8008583	q12
AB	BB	AA	NoCall	AA	AA	Exclude	rs9788516	q12

Chromosome 1 comparison:

III:3	IV:4	II:3	III:7	IV:6	IV:7	MCDR4 AD	dbSNP RS ID	Cytoband	III:3	IV:4	II:3	III:7	IV:6	IV:7	MCDR4 AD	dbSNP RS ID	Cytoband
BB	BB	BB	BB	BB	BB	BB	rs17471243	p36.31	BB	BB	AB	NoCall	AB	AB	BB	rs9434834	p36.31
NoCall	BB	BB	BB	AB	AB	BB	rs11121600	p36.31	BB	BB	BB	NoCall	BB	BB	BB	rs10864279	p36.31
AB	BB	NoCall	AB	AB	NoCall	BB	rs11121605	p36.31	NoCall	AB	NoCall	NoCall	NoCall	NoCall	AB	rs11120840	p36.31
AA	AA	AA	AB	AA	AA	AA	rs11121648	p36.31	AB	AB	AB	AB	AA	AA	AA	rs10779675	p36.23
AB	AA	AA	AA	AB	AB	AA	rs489933	p36.31	AB	AB	BB	BB	BB	BB	BB	rs6656160	p36.23
AA	AA	AA	AA	AA	AA	AA	rs12737596	p36.31	NoCall	BB	BB	BB	BB	BB	BB	rs17030341	p36.23
AB	BB	BB	BB	BB	BB	BB	rs4908559	p36.31	AB	AB	AB	AA	AA	AA	AA	rs7527889	p36.23
AA	AA	AA	NoCall	AB	AB	AA	rs2312464	p36.31	AB	AB	AB	BB	AB	AB	BB	rs11120869	p36.23
NoCall	NoCall	BB	NoCall	AB	AB	BB	rs12120967	p36.31	AB	AB	AB	AA	AB	AB	AA	rs6680365	p36.23
BB	BB	BB	NoCall	BB	BB	BB	rs12093500	p36.31	NoCall	AA	AB	BB	AB	AB	Exclude	rs4908607	p36.23
AB	BB	BB	BB	BB	BB	BB	rs3747987	p36.31	NoCall	AA	AB	BB	AB	AB	Exclude	rs2157499	p36.23
AA	AA	NoCall	AA	AA	AA	AA	rs11122130	p36.31	BB	BB	BB	BB	BB	BB	BB	rs17030589	p36.23
AA	AA	AA	AA	NoCall	AB	AA	rs12123214	p36.31	AA	AA	AA	NoCall	NoCall	AA	AA	rs766156	p36.23
AA	AA	AA	AA	AA	AA	AA	rs17028879	p36.31	AA	NoCall	AA	AA	AA	AA	AA	rs12144179	p36.23
NoCall	BB	NoCall	BB	NoCall	BB	BB	rs6694744	p36.31	AB	AA	AB	BB	NoCall	AB	Exclude	rs1476047	p36.23
BB	BB	BB	BB	AB	AB	BB	rs1011994	p36.31	BB	BB	BB	BB	BB	BB	BB	rs6577427	p36.23
AA	AA	AA	AA	AB	AB	AA	rs11576617	p36.31	BB	AB	NoCall	AB	AB	AB	BB	rs7549870	p36.23
BB	BB	BB	BB	BB	BB	BB	rs10492950	p36.31	BB	AB	BB	NoCall	AB	NoCall	BB	rs7515140	p36.23
AA	AA	AA	AA	AB	AB	AA	rs10864284	p36.31	BB	NoCall	AB	NoCall	AA	AA	Exclude	rs4908612	p36.23
AA	NoCall	NoCall	NoCall	AB	AB	AA	rs6659420	p36.31	NoCall	NoCall	AA	AA	NoCall	AA	AA	rs1034453	p36.23
AA	AA	AA	NoCall	AB	AB	AA	rs4068399	p36.31	AA	AB	AA	AA	NoCall	AA	AA	rs7517429	p36.23
NoCall	NoCall	NoCall	AA	AA	AA	AA	rs4908639	p36.31	BB	AB	BB	BB	BB	BB	BB	rs1034454	p36.23
BB	BB	BB	BB	BB	BB	BB	rs709207	p36.31	AB	BB	AB	AA	AB	AB	Exclude	rs11120896	p36.23
BB	BB	BB	NoCall	NoCall	AB	BB	rs1295101	p36.31	NoCall	AA	AA	AA	AA	AA	AA	rs2301462	p36.23
AA	AA	AA	AA	AA	AA	AA	rs7524578	p36.31	AA	AA	AA	AA	AA	AA	AA	rs10864294	p36.23
AB	BB	AB	AA	AA	AA	Exclude	rs806114	p36.31	AA	AB	AA	AA	AA	AA	AA	rs705681	p36.23
NoCall	BB	NoCall	BB	NoCall	BB	BB	rs17029061	p36.31	AA	AB	AA	AA	AA	AA	AA	rs705680	p36.23
BB	NoCall	BB	NoCall	NoCall	NoCall	BB	rs1295103	p36.31	BB	AB	BB	BB	BB	BB	BB	rs845244	p36.23
AA	AA	AA	NoCall	AA	NoCall	AA	rs12047044	p36.31	AA	AA	AA	AA	AA	AA	AA	rs4908456	p36.23
AA	AA	AA	AA	AA	NoCall	AA	rs1883771	p36.31	AA	NoCall	AA	NoCall	NoCall	NoCall	AA	rs845240	p36.23
AA	AA	AA	NoCall	NoCall	AA	AA	rs6701690	p36.31	AA	AB	AA	AB	AA	AA	AA	rs4908624	p36.23
AB	AA	AA	AA	NoCall	AA	AA	rs3789541	p36.31	BB	BB	BB	NoCall	BB	BB	BB	rs11120905	p36.23
AA	NoCall	AA	AA	AA	NoCall	AA	rs3789530	p36.31	AA	AA	AA	NoCall	AA	NoCall	AA	rs845196	p36.23
BB	BB	NoCall	AA	AB	AB	Exclude	rs1883606	p36.31	BB	BB	NoCall	AB	BB	NoCall	BB	rs845195	p36.23
NoCall	AA	NoCall	AA	AA	NoCall	AA	rs12071731	p36.31	BB	BB	BB	AB	BB	BB	BB	rs845194	p36.23
BB	BB	BB	BB	BB	NoCall	BB	rs1064721	p36.31	BB	BB	BB	AB	BB	BB	BB	rs705672	p36.23
AA	AA	AA	AA	AA	AA	AA	rs14281	p36.31	NoCall	NoCall	AA	AA	AA	AA	AA	rs1418489	p36.23
AB	BB	AB	AA	AA	AA	Exclude	rs2057006	p36.31	AA	AA	AA	AB	AA	AA	AA	rs845265	p36.23
NoCall	BB	BB	BB	BB	NoCall	BB	rs11811690	p36.31	AA	AA	AA	AA	AA	AA	AA	rs3752543	p36.23
BB	NoCall	BB	BB	BB	BB	BB	rs10489535	p36.31	BB	BB	BB	AB	BB	BB	BB	rs845218	p36.23
BB	BB	BB	BB	NoCall	AB	BB	rs35553413	p36.31	BB	BB	NoCall	AB	BB	NoCall	BB	rs845208	p36.23
NoCall	BB	NoCall	BB	NoCall	AB	BB	rs7554077	p36.31	BB	BB	BB	NoCall	BB	BB	BB	rs4908629	p36.23
NoCall	NoCall	BB	BB	AB	AB	BB	rs3789492	p36.31	BB	BB	BB	AB	BB	NoCall	BB	rs845232	p36.23
BB	BB	BB	BB	AB	AB	BB	rs3789484	p36.31	BB	BB	BB	NoCall	BB	BB	BB	rs12088178	p36.23
BB	NoCall	BB	BB	NoCall	BB	BB	rs2010905	p36.31	NoCall	AA	AA	AA	AB	AB	AA	rs1193221	p36.23
AA	AA	AA	AA	AB	AB	AA	rs9662106	p36.31	AA	NoCall	AA	NoCall	NoCall	NoCall	AA	rs17030881	p36.23
BB	BB	NoCall	BB	BB	BB	BB	rs3789470	p36.31	BB	BB	BB	AB	AB	BB	BB	rs1193247	p36.23
BB	BB	BB	BB	AB	AB	BB	rs11364	p36.31	AB	NoCall	AB	NoCall	AA	AA	AA	rs1880808	p36.23
BB	BB	BB	BB	AB	AB	BB	rs10864548	p36.31	NoCall	BB	NoCall	BB	BB	BB	BB	rs17030898	p36.23
AA	AA	AA	AA	NoCall	AB	AA	rs3007421	p36.31	BB	BB	NoCall	NoCall	NoCall	BB	BB	rs732400	p36.23
NoCall	AA	AB	AB	NoCall	NoCall	AA	rs2986739	p36.31	AA	AA	AA	AA	AA	AA	AA	rs732401	p36.23
AB	AB	AB	AB	AB	AB	AB	rs4908901	p36.31	BB	BB	BB	BB	BB	BB	BB	rs17030961	p36.23
AA	AA	AA	AA	AA	AA	AA	rs4509598	p36.31	BB	BB	BB	BB	BB	BB	BB	rs7526892	p36.23
NoCall	BB	BB	BB	BB	BB	BB	rs7548042	p36.31	AB	AB	AB	AB	AB	AB	AB	rs1750849	p36.23
NoCall	AA	NoCall	NoCall	NoCall	NoCall	AA	rs11122082	p36.31	AA	NoCall	AA	NoCall	NoCall	AA	AA	rs10864304	p36.23
NoCall	NoCall	NoCall	NoCall	AB	AB	AB	rs6679649	p36.31	AA	AA	AA	AA	AA	AA	AA	rs11120958	p36.23
BB	BB	BB	BB	AB	AB	BB	rs10746502	p36.31	AB	AB	AB	AA	AB	NoCall	AA	rs6577446	p36.23
BB	BB	BB	NoCall	NoCall	BB	BB	rs10864624	p36.31	AA	AB	AB	AB	BB	BB	Exclude	rs2177901	p36.23
BB	NoCall	BB	BB	NoCall	BB	BB	rs6577582	p36.31	AA	AA	AA	AA	AA	AA	AA	rs17031077	p36.23
AB	BB	BB	BB	BB	BB	BB	rs731024	p36.31	AB	AA	AA	AB	AA	AA	AA	rs11120970	p36.23
BB	BB	BB	BB	BB	NoCall	BB	rs200457	p36.31	BB	BB	BB	BB	BB	BB	BB	rs6696544	p36.23
NoCall	AB	BB	AB	NoCall	BB	BB	rs2235564	p36.31	AA	AA	AA	AA	AA	AA	AA	rs17031145	p36.23
AB	BB	BB	AB	BB	BB	BB	rs200440	p36.31	BB	BB	BB	BB	BB	BB	BB	rs12059360	p36.23
AB	AA	AA	AB	AA	AA	AA	rs3827729	p36.31	AA	NoCall	AA	AA	AA	NoCall	AA	rs11120978	p36.23
AB	AA	NoCall	AB	AA	AA	AA	rs11122119	p36.31	BB	BB	BB	BB	BB	BB	BB	rs12076773	p36.23
AA	AA	AA	AA	AA	AA	AA	rs277671	p36.31	BB	BB	BB	BB	BB	BB	BB	rs6683993	p36.23
AB	AA	AA	AB	AA	AA	AA	rs277672	p36.31	AB	NoCall	AB	AA	NoCall	AA	AA	rs12139380	p36.23
AB	AA	AA	AB	AA	AA	AA	rs277673	p36.31	AB	AB	AB	AA	AA	AA	AA	rs12139470	p36.23
NoCall	AA	AA	NoCall	AA	AA	AA	rs17027008	p36.31	AB	AB	AB	AA	AA	AA	AA	rs12139551	p36.23
BB	BB	BB	AB	BB	BB	BB	rs448886	p36.31	NoCall	AB	AB	AA	AA	NoCall	AA	rs17031211	p36.23
BB	AB	BB	NoCall	NoCall	NoCall	BB	rs950493	p36.31	BB	BB	AB	AA	AA	AA	Exclude	rs4908660	p36.23
AA	AB	AA	AA	NoCall	AA	AA	rs12727063	p36.31	NoCall	NoCall	BB	BB	NoCall	BB	BB	rs4908473	p36.23
AA	AA	AA	AA	AA	AA	AA	rs17030041	p36.31	BB	BB	BB	BB	BB	NoCall	BB	rs6658296	p36.23
AB	AA	AA	AA	AA	AA	AA	rs4908426	p36.31	AA	AB	AB	AB	AB	AB	AA	rs3011926	p36.23
NoCall	NoCall	NoCall	NoCall	NoCall	NoCall	AB	rs7519035	p36.31	BB	BB	BB	BB	BB	BB	BB	rs947417	p36.23
AB	BB	BB	AB	NoCall	NoCall	BB	rs6577395	p36.31	AB	BB	AB	AB	AB	AB	BB	rs2995032	p36.23
AB	AA	AA	AB	AA	AA	AA	rs4908575	p36.31	AB	BB	AB	AB	AB	AB	BB	rs2995034	p36.23
AB	AA	AA	AB	AA	AA	AA	rs12134815	p36.31	BB	BB	BB	BB	BB	BB	BB	rs11121007	p36.23
AB	BB	NoCall	AB	BB	BB	BB	rs7538627	p36.31	AA	AA	AA	AA	AA	AA	AA	rs4908688	p36.23
AB	AA	AA	AB	AA	AA	AA	rs10864255	p36.31	BB	BB	BB	BB	BB	BB	BB	rs11121014	p36.23
AA	AA	NoCall	AA	NoCall	AA	AA	rs11120797	p36.31	NoCall	AB	AB	BB	BB	NoCall	BB	rs1417986	p36.23
AA	AA	AB	BB	AB	AB	Exclude	rs1013248	p36.31	BB	BB	BB	BB	BB	BB	BB	rs9919220	p36.23
AA	AA	AA	AA	AA	AA	AA	rs11120799	p36.31	NoCall	NoCall	BB	BB	BB	BB	BB	rs12093175	p36.23
BB	NoCall	AB	AA	AB	AB	Exclude	rs17030082	p36.31	BB	BB	AB	AA	AA	AA	Exclude	rs707458	p36.23
NoCall	AA	AB	BB	AB	NoCall	Exclude	rs6679134	p36.31	NoCall	AA	AA	NoCall	AA	AA	AA	rs4908694	p36.23
AB	BB	NoCall	AA	NoCall	AB	Exclude	rs4518923	p36.31	NoCall	BB	NoCall	BB	BB	BB	BB	rs1012477	p36.23
AB	AA	AB	BB	BB	BB	Exclude	rs6701312	p36.31	NoCall	BB	BB	NoCall	AB	AB	BB	rs2859389	p36.23
AB	AA	AB	BB	BB	BB	Exclude	rs6698901	p36.31	AB	AB	BB	AB	AB	AB	BB	rs227147	p36.23
BB	NoCall	NoCall	NoCall	NoCall	NoCall	BB	rs12061141	p36.31	AA	AA	AA	AA	AA	AA	AA	rs12068202	p36.23
AB	AB	AB	BB	BB	BB	BB	rs6656639	p36.31	AA	AA	AA	AA	AA	AA	AA	rs161803	p36.23
AB	AB	AB	AA	AA	AA	AA	rs11120818	p36.31	BB	BB	BB	BB	BB	BB	BB	rs9657980	p36.23
BB	AB	BB	BB	BB	BB	BB	rs12048981	p36.31	BB	BB	BB	BB	NoCall	BB	BB	rs9657961	p36.23
AB	AA	AB	BB	AB	AB	Exclude	rs7517585	p36.31	BB	BB	NoCall	BB	NoCall	BB	BB	rs161811	p36.23
AB	NoCall	AB	NoCall	NoCall	AB	AB	rs6690314	p36.31	BB	BB	NoCall	BB	NoCall	BB	BB	rs226473	p36.23
AB	BB	AB	AA	AB	AB	Exclude	rs7553100	p36.31	NoCall	NoCall	NoCall	NoCall	NoCall	BB	BB	rs161801	p36.23
NoCall	BB	BB	NoCall	NoCall	NoCall	BB	rs3923058	p36.31	BB	BB	BB	AB	BB	BB	BB	rs17367289	p36.23
BB	AB	BB	BB	AB	AB	BB	rs4908596	p36.31	AA	NoCall	AA	AB	AA	NoCall	AA	rs4908727	p36.23
NoCall	NoCall	AA	NoCall	NoCall	AA	AA	rs11120837	p36.31	AA	NoCall	AA	AB	AA	NoCall	AA	rs7545677	p36.23
BB	AB	BB	BB	BB	BB	BB	rs7511741	p36.31	BB	BB	BB	AB	BB	BB	BB	rs6703955	p36.23
BB	BB	BB	BB	BB	BB	BB	rs7515073	p36.31	BB	BB	AB	AB	AB	AB	BB	rs11121090	p36.23
BB	BB	AB	NoCall	AB	AB	BB	rs9434834	p36.31	AA	AA	AA	AB	NoCall	NoCall	AA	rs4369247	p36.23

Chromosome 5 comparison:

III:3	IV:4	II:3	III:7	IV:6	IV:7	MCDR4 AD	dbSNP RS ID	Cytoband	III:3	IV:4	II:3	III:7	IV:6	IV:7	MCDR4 AD	dbSNP RS ID	Cytoband
AB	NoCall	BB	BB	BB	BB	BB	rs16878933	p15.33	AA	AA	AA	AA	AA	AA	AA	rs316589	p15.33
BB	BB	BB	BB	NoCall	BB	BB	rs17588991	p15.33	AB	AB	AA	AA	AA	AA	AA	rs898061	p15.33
BB	BB	BB	BB	BB	BB	BB	rs2962041	p15.33	NoCall	AA	AB	AB	BB	BB	Exclude	rs1545899	p15.33
BB	AB	BB	AB	AB	AB	BB	rs4398676	p15.33	AB	BB	AB	AB	AA	AA	Exclude	rs2628163	p15.33
NoCall	NoCall	BB	NoCall	NoCall	BB	BB	rs7700579	p15.33	AB	AA	AB	AB	BB	BB	Exclude	rs13169221	p15.33
NoCall	AB	AA	AA	AA	AA	AA	rs7712396	p15.33	NoCall	NoCall	AA	NoCall	NoCall	NoCall	AA	rs408776	p15.33
BB	NoCall	NoCall	BB	BB	NoCall	BB	rs2963266	p15.33	BB	BB	BB	BB	BB	BB	BB	rs462894	p15.33
NoCall	BB	AB	NoCall	AB	BB	BB	rs10866513	p15.33	AB	BB	BB	BB	BB	BB	BB	rs16899400	p15.33
AA	AA	NoCall	AA	AA	AA	AA	rs16880246	p15.33	AB	BB	BB	AB	AB	AB	BB	rs465850	p15.33
AA	AA	AA	AA	AA	AA	AA	rs17596634	p15.33	AB	AA	NoCall	AB	AB	AB	AA	rs462203	p15.33
AA	AA	AA	NoCall	NoCall	AA	AA	rs1054540	p15.33	BB	AB	BB	BB	BB	BB	BB	rs650640	p15.33
AA	AA	AA	AA	AA	AA	AA	rs4975681	p15.33	BB	BB	BB	BB	NoCall	BB	BB	rs16869166	p15.33
BB	NoCall	NoCall	NoCall	AB	NoCall	BB	rs6554915	p15.33	NoCall	AB	NoCall	AB	AB	AB	AB	rs11952610	p15.33
AA	NoCall	AA	NoCall	NoCall	AA	AA	rs10475076	p15.33	AA	AB	AA	AB	AB	AB	AA	rs4866643	p15.33
AA	AA	AA	AA	AB	AA	AA	rs4424035	p15.33	NoCall	NoCall	BB	NoCall	NoCall	AB	BB	rs4866483	p15.33
NoCall	BB	NoCall	BB	NoCall	NoCall	BB	rs6897438	p15.33	NoCall	BB	BB	BB	BB	BB	BB	rs16869189	p15.33
BB	BB	BB	BB	BB	BB	BB	rs10079800	p15.33	NoCall	NoCall	AA	AB	AB	NoCall	AA	rs17648791	p15.33
NoCall	BB	BB	BB	BB	BB	BB	rs6882694	p15.33	AA	AB	AA	AB	AB	AB	AA	rs4866484	p15.33
BB	NoCall	BB	BB	BB	BB	BB	rs16883175	p15.33	NoCall	AB	NoCall	AB	AB	AB	AB	rs315914	p15.33
AA	AA	AA	AA	AA	AA	AA	rs16883213	p15.33	BB	NoCall	BB	NoCall	AB	AB	BB	rs370672	p15.33
BB	NoCall	AB	BB	NoCall	BB	BB	rs17599063	p15.33	AB	NoCall	BB	NoCall	AA	AA	Exclude	rs6555108	p15.33
AB	BB	BB	BB	NoCall	BB	BB	rs872015	p15.33	AB	BB	AB	AB	AA	AB	Exclude	rs1908159	p15.33
BB	BB	BB	BB	BB	BB	BB	rs16883787	p15.33	BB	NoCall	BB	BB	BB	BB	BB	rs10070890	p15.33
AA	AA	AA	AA	AA	AA	AA	rs16884043	p15.33	AB	BB	BB	NoCall	AB	AB	BB	rs6880842	p15.33
BB	BB	BB	BB	BB	BB	BB	rs904759	p15.33	NoCall	BB	BB	BB	NoCall	BB	BB	rs6876992	p15.33
NoCall	AA	AA	NoCall	NoCall	NoCall	AA	rs6895045	p15.33	BB	BB	BB	NoCall	NoCall	BB	BB	rs1873955	p15.33
AB	AA	AB	AB	BB	AB	Exclude	rs6554987	p15.33	AB	AA	AB	AB	AB	AB	AA	rs2453814	p15.33
AA	AA	NoCall	AA	AA	AA	AA	rs1392466	p15.33	AB	AA	AB	AB	AB	AB	AA	rs895658	p15.33
NoCall	AA	AA	NoCall	AA	AA	AA	rs1392469	p15.33	AA	AA	AA	AA	AA	AA	AA	rs2218593	p15.33
AA	AA	AA	AA	AA	AA	AA	rs10040260	p15.33	BB	BB	BB	BB	BB	BB	BB	rs6896543	p15.33
AB	BB	BB	NoCall	AB	BB	BB	rs904756	p15.33	AB	BB	AB	AB	AB	AB	BB	rs453357	p15.33
BB	BB	AB	BB	BB	AB	BB	rs16884579	p15.33	AB	BB	AB	AB	AB	AB	BB	rs11744790	p15.33
AA	AA	AB	AA	AA	AB	AA	rs5000660	p15.33	AB	BB	AB	AB	AB	AB	BB	rs11133954	p15.33
NoCall	AA	NoCall	AA	AA	AA	AA	rs904757	p15.33	AB	BB	AB	NoCall	NoCall	AB	BB	rs11748913	p15.33
AA	AA	AA	AA	AA	AA	AA	rs904758	p15.33	AA	AA	AA	AA	AA	AA	AA	rs13355848	p15.33
BB	BB	BB	BB	BB	NoCall	BB	rs9312955	p15.33	NoCall	AB	NoCall	BB	BB	NoCall	BB	rs7729915	p15.33
BB	BB	NoCall	NoCall	NoCall	BB	BB	rs7711522	p15.33	AA	AA	AA	AA	AA	AA	AA	rs16869936	p15.33
BB	BB	BB	BB	BB	BB	BB	rs4975845	p15.33	BB	AB	BB	BB	NoCall	NoCall	BB	rs9313003	p15.33
AA	AA	AA	NoCall	AA	AA	AA	rs7702843	p15.33	BB	BB	NoCall	NoCall	AA	AA	Exclude	rs424339	p15.33
BB	BB	AB	NoCall	AB	BB	BB	rs4588629	p15.33	AB	AA	AA	AA	AA	AA	AA	rs16870028	p15.33
NoCall	AA	NoCall	AA	NoCall	NoCall	AA	rs10085011	p15.33	NoCall	AA	AB	AB	BB	BB	Exclude	rs404016	p15.33
NoCall	AA	AB	AA	AB	AA	AA	rs4304110	p15.33	AA	AA	AA	AB	BB	BB	Exclude	rs434958	p15.33
BB	BB	BB	BB	BB	BB	BB	rs4975731	p15.33	NoCall	BB	NoCall	AB	AA	AA	Exclude	rs380259	p15.33
AA	AA	AB	AA	AB	AA	AA	rs17535721	p15.33	NoCall	AA	NoCall	NoCall	AA	AA	AA	rs435098	p15.33
BB	BB	BB	BB	BB	BB	BB	rs10078791	p15.33	AA	AA	AA	AB	BB	BB	Exclude	rs6893795	p15.33
AA	AA	AB	AA	AB	NoCall	AA	rs4975732	p15.33	NoCall	BB	NoCall	AB	AB	AB	BB	rs225752	p15.33
BB	BB	BB	BB	BB	NoCall	BB	rs6875087	p15.33	BB	BB	AB	BB	BB	BB	BB	rs7726088	p15.33
AA	AA	NoCall	AA	AA	AA	AA	rs6555013	p15.33	NoCall	NoCall	AB	AA	AA	NoCall	AA	rs7702236	p15.33
BB	BB	AB	BB	AB	BB	BB	rs6873986	p15.33	AB	AA	AB	AA	AA	AB	AA	rs2004187	p15.33
NoCall	AA	AA	AA	AA	AA	AA	rs16901230	p15.33	AA	AA	NoCall	AA	AA	AA	AA	rs9313007	p15.33
AB	BB	AB	AB	AB	AA	Exclude	rs10512645	p15.33	AB	AA	AB	AA	AA	AB	AA	rs2441942	p15.33
AB	AA	AB	AA	AA	AB	AA	rs4975741	p15.33	BB	BB	BB	NoCall	BB	BB	BB	rs16870184	p15.33
AB	NoCall	AB	AA	AA	AB	AA	rs1532272	p15.33	BB	NoCall	BB	BB	BB	BB	BB	rs16870220	p15.33
AA	AA	AA	AB	BB	AB	Exclude	rs4975744	p15.33	AA	AB	AB	AA	AB	AB	AA	rs323660	p15.33
NoCall	NoCall	AA	AA	NoCall	AA	AA	rs1684969	p15.33	BB	AB	BB	BB	BB	BB	BB	rs17608672	p15.33
AA	AA	AA	NoCall	AA	NoCall	AA	rs1689747	p15.33	AA	NoCall	AA	AA	AA	AA	AA	rs1393235	p15.33
AA	AA	AA	AA	AA	AA	AA	rs2353580	p15.33	AA	AB	AB	AA	AA	AB	AA	rs2962558	p15.33
AA	AA	AA	AB	BB	AB	Exclude	rs883823	p15.33	BB	NoCall	BB	BB	BB	AB	BB	rs2937761	p15.33
AA	AA	NoCall	AA	AB	AA	AA	rs4975752	p15.33	BB	NoCall	BB	NoCall	NoCall	NoCall	BB	rs950865	p15.33
NoCall	NoCall	NoCall	NoCall	NoCall	BB	BB	rs260406	p15.33	AA	AA	AA	AA	AA	AB	AA	rs10042330	p15.33
AB	AB	AB	AB	BB	BB	BB	rs17537048	p15.33	NoCall	BB	BB	BB	AB	AB	BB	rs10054312	p15.33
AB	AB	AA	AB	NoCall	AB	AA	rs10042507	p15.33	AB	AA	AA	AA	AB	AA	AA	rs7704762	p15.33
AA	AB	AA	AB	AB	NoCall	AA	rs11744276	p15.33	NoCall	NoCall	AA	AA	AA	AB	AA	rs2935620	p15.33
AB	AB	AB	AB	AB	AB	AB	rs7737997	p15.33	AA	AA	AA	AA	NoCall	AB	AA	rs2935621	p15.33
NoCall	AB	AA	AA	AB	NoCall	AA	rs4975775	p15.33	AA	AA	AB	AB	AB	BB	Exclude	rs2935624	p15.33
AA	AB	AA	AA	AB	AB	AA	rs1123381	p15.33	BB	BB	NoCall	NoCall	BB	BB	BB	rs16870285	p15.33
AA	AB	AA	AA	AB	AB	AA	rs1123391	p15.33	NoCall	NoCall	NoCall	NoCall	NoCall	NoCall	AB	rs16870313	p15.33
BB	AB	NoCall	BB	AB	AB	BB	rs1123390	p15.33	AA	AA	AB	AB	AB	BB	Exclude	rs904124	p15.33
BB	BB	AB	AB	AB	AB	BB	rs10043083	p15.33	BB	BB	BB	BB	BB	BB	BB	rs875221	p15.33
BB	AB	AB	AB	AA	AA	Exclude	rs2101404	p15.33	BB	BB	BB	BB	BB	AB	BB	rs11133959	p15.33
AA	NoCall	AA	AA	AA	AA	AA	rs746365	p15.33	AA	AA	AB	AB	AB	BB	Exclude	rs958582	p15.33
AA	AB	AB	AB	BB	BB	Exclude	rs11747860	p15.33	AA	AA	AB	AB	AB	BB	Exclude	rs2962587	p15.33
BB	BB	BB	AB	AA	AA	Exclude	rs13190016	p15.33	BB	BB	AB	AB	AB	AA	Exclude	rs2935637	p15.33
AB	AA	AB	AA	AA	AA	AA	rs4591793	p15.33	BB	BB	AB	AB	AB	AB	BB	rs2935644	p15.33
NoCall	NoCall	NoCall	NoCall	AA	AA	AA	rs4075367	p15.33	BB	NoCall	BB	BB	NoCall	BB	BB	rs6862975	p15.33
AB	BB	AB	BB	BB	BB	BB	rs11952498	p15.33	AA	AA	AB	AB	AB	AB	AA	rs2962580	p15.33
AA	AA	AA	AA	AA	AA	AA	rs11960538	p15.33	AA	AA	AA	AA	AA	AA	AA	rs2132868	p15.33
AA	AA	AA	AA	AA	AA	AA	rs7448302	p15.33	AB	AA	AB	AA	AB	AB	AA	rs2047382	p15.33
AA	AA	AA	AA	AA	NoCall	AA	rs11133930	p15.33	AA	AA	AA	AA	NoCall	NoCall	AA	rs1566160	p15.33
AA	AA	AA	AA	AA	AA	AA	rs16903364	p15.33	AB	BB	AB	NoCall	NoCall	AB	BB	rs4866666	p15.33
AB	AB	BB	NoCall	AB	AB	BB	rs17583962	p15.33	NoCall	BB	NoCall	NoCall	NoCall	NoCall	BB	rs1497464	p15.33
BB	BB	BB	BB	BB	BB	BB	rs16903407	p15.33	NoCall	AA	AA	AA	NoCall	AA	AA	rs1497463	p15.33
AB	AB	BB	NoCall	AB	AB	BB	rs13171126	p15.33	AB	AB	NoCall	NoCall	NoCall	AB	AB	rs11952868	p15.33
NoCall	NoCall	NoCall	NoCall	NoCall	AA	AA	rs6863477	p15.33	BB	BB	BB	NoCall	NoCall	BB	BB	rs6864829	p15.33
AB	AB	AA	AB	AB	AB	AA	rs3923314	p15.33	BB	AB	BB	AB	NoCall	AB	BB	rs6867209	p15.33
BB	BB	AB	BB	BB	AB	BB	rs4866625	p15.33	NoCall	AB	BB	AB	AB	AB	BB	rs1908976	p15.33
BB	BB	AB	BB	BB	AB	BB	rs11133935	p15.33	AA	AB	AA	AB	AB	AB	AA	rs1908975	p15.33
AB	AB	NoCall	AB	AB	AB	AB	rs6861416	p15.33	BB	AB	BB	AB	AB	AB	BB	rs1908974	p15.33
NoCall	NoCall	AA	NoCall	NoCall	NoCall	AA	rs16903629	p15.33	AB	NoCall	BB	BB	AB	NoCall	BB	rs16870454	p15.33
AA	AA	AB	AA	AA	AB	AA	rs10041522	p15.33	AB	NoCall	BB	BB	NoCall	BB	BB	rs10053398	p15.33
AA	AA	AB	NoCall	NoCall	AB	AA	rs17646078	p15.33	AA	AA	AA	AA	AA	AA	AA	rs16870456	p15.33
AA	AA	AB	AA	AA	AA	AA	rs11954767	p15.33	BB	AB	BB	AB	AB	AB	BB	rs4866499	p15.33
AA	AA	AB	AA	AA	AA	AA	rs11952611	p15.33	BB	BB	BB	AB	AB	AA	Exclude	rs4866504	p15.33
AA	NoCall	AA	AA	NoCall	AA	AA	rs563704	p15.33	AB	BB	BB	BB	BB	BB	BB	rs1122217	p15.33
AA	AB	AA	AB	BB	AB	Exclude	rs635969	p15.33	NoCall	BB	BB	BB	NoCall	BB	BB	rs1476289	p15.33
BB	BB	BB	BB	BB	BB	BB	rs3849693	p15.33	NoCall	NoCall	BB	BB	NoCall	BB	BB	rs16870518	p15.33
BB	BB	BB	BB	BB	BB	BB	rs3931986	p15.33	AB	AA	AA	AA	AA	AA	AA	rs13184825	p15.33
AA	AA	AA	AA	AB	AA	AA	rs6898118	p15.33	BB	NoCall	NoCall	NoCall	NoCall	NoCall	BB	rs1018089	p15.33
NoCall	AA	AA	AA	AA	AA	AA	rs7718226	p15.33	AA	AA	AA	AA	AA	AA	AA	rs6862810	p15.33
AA	AB	AA	AA	AA	AA	AA	rs117622	p15.33	NoCall	BB	AB	NoCall	NoCall	BB	BB	rs4866685	p15.33
AA	AA	AA	AA	AA	AA	AA	rs316589	p15.33	BB	BB	AB	BB	BB	BB	BB	rs6890790	p15.33

Chromosome 16 comparison:

III:3	IV:4	II:3	III:7	IV:6	IV:7	MCDR4 AD	dbSNP RS ID	Cytoband	III:3	IV:4	II:3	III:7	IV:6	IV:7	MCDR4 AD	dbSNP RS ID	Cytoband
AB	AB	AA	AA	AA	AB	AA	rs6600227	p13.3	NoCall	BB	NoCall	BB	AB	NoCall	BB	rs10903010	p13.3
NoCall	AA	AA	AA	AA	AA	AA	rs1366553	p13.3	NoCall	NoCall	NoCall	AA	AB	AA	AA	rs9927202	p13.3
BB	BB	BB	BB	NoCall	NoCall	BB	rs216612	p13.3	NoCall	BB	BB	BB	NoCall	BB	BB	rs7196996	p13.3
BB	BB	BB	BB	BB	BB	BB	rs3176398	p13.3	AA	AA	AA	AA	AB	NoCall	AA	rs9940288	p13.3
AA	AA	AA	NoCall	NoCall	NoCall	AA	rs3176406	p13.3	AA	AA	AA	AA	AB	AA	AA	rs7199342	p13.3
BB	BB	BB	BB	BB	BB	BB	rs2562181	p13.3	AA	AA	AA	NoCall	NoCall	AA	AA	rs34148924	p13.3
BB	AB	AB	AB	AB	AB	BB	rs6600233	p13.3	AA	AA	AA	AA	AA	AA	AA	rs17135510	p13.3
NoCall	AA	AA	AA	AA	AA	AA	rs17525396	p13.3	AB	AA	AA	AA	AA	AB	AA	rs2575369	p13.3
AA	NoCall	NoCall	AA	AA	NoCall	AA	rs2541615	p13.3	AA	AA	AA	AA	AA	AA	AA	rs9926962	p13.3
AB	AB	AB	AB	AB	AA	AA	rs798612	p13.3	NoCall	AA	AA	AA	AA	AB	AA	rs1028590	p13.3
NoCall	NoCall	BB	BB	BB	BB	BB	rs2238367	p13.3	NoCall	NoCall	NoCall	NoCall	NoCall	NoCall	AB	rs8045169	p13.3
AB	AB	AB	NoCall	AB	AA	AA	rs216091	p13.3	BB	BB	BB	NoCall	BB	AB	BB	rs1625279	p13.3
AB	AB	NoCall	AB	AB	NoCall	AB	rs183350	p13.3	AA	AA	AA	AA	AA	AB	AA	rs1625393	p13.3
BB	BB	BB	BB	BB	BB	BB	rs177510	p13.3	BB	BB	BB	BB	BB	AB	BB	rs1657117	p13.3
NoCall	AB	AB	AB	AB	NoCall	AB	rs216093	p13.3	BB	BB	BB	BB	BB	AB	BB	rs4420529	p13.3
AA	AA	AA	AA	AA	AA	AA	rs798604	p13.3	AA	AA	AA	AA	AA	AA	AA	rs2429186	p13.3
BB	BB	BB	BB	BB	BB	BB	rs7198876	p13.3	NoCall	BB	AA	NoCall	BB	BB	Exclude	rs2252523	p13.3
NoCall	NoCall	BB	AB	AA	AA	Exclude	rs6600143	p13.3	BB	BB	BB	BB	BB	BB	BB	rs8053843	p13.3
NoCall	AA	AA	AB	AB	BB	Exclude	rs2541639	p13.3	AA	AA	AA	AA	AA	AA	AA	rs17602	p13.3
AB	AA	AA	AA	AB	AA	AA	rs2974771	p13.3	BB	BB	AB	BB	BB	BB	BB	rs2240690	p13.3
AB	AB	AB	BB	AB	AB	BB	rs1211375	p13.3	NoCall	AA	NoCall	NoCall	AA	NoCall	AA	rs8051877	p13.3
AB	AB	AB	NoCall	AA	AB	AA	rs11248914	p13.3	BB	BB	AB	BB	BB	BB	BB	rs2516739	p13.3
AB	NoCall	AB	BB	NoCall	AB	BB	rs11248915	p13.3	AA	NoCall	AA	NoCall	NoCall	NoCall	AA	rs8059880	p13.3
AB	AA	AA	AA	AA	AA	AA	rs9940149	p13.3	NoCall	BB	AB	BB	NoCall	BB	BB	rs2516737	p13.3
AA	AA	AA	AA	AA	NoCall	AA	rs3743878	p13.3	BB	BB	AA	AA	AB	NoCall	Exclude	rs1640778	p13.3
BB	NoCall	BB	BB	BB	NoCall	BB	rs17136027	p13.3	AA	AA	AA	AA	AA	AA	AA	rs150928	p13.3
AA	NoCall	AA	AA	AB	AA	AA	rs8063821	p13.3	BB	NoCall	NoCall	NoCall	AB	AB	BB	rs2014467	p13.3
BB	BB	BB	NoCall	NoCall	BB	BB	rs11864322	p13.3	AB	AA	NoCall	AA	NoCall	AA	AA	rs323073	p13.3
AA	AA	NoCall	NoCall	AA	AA	AA	rs17136056	p13.3	AB	AA	AB	AA	AA	AA	AA	rs11867129	p13.3
AA	AA	NoCall	NoCall	AA	AA	AA	rs9746347	p13.3	BB	BB	BB	BB	NoCall	NoCall	BB	rs34158278	p13.3
AA	AA	AA	AA	AA	AA	AA	rs17136099	p13.3	AA	NoCall	AA	NoCall	NoCall	AA	AA	rs323064	p13.3
AA	AA	AA	AA	AA	AA	AA	rs17136103	p13.3	AA	AA	AA	AA	AA	AA	AA	rs323052	p13.3
AB	NoCall	AA	AA	AB	AA	AA	rs9925273	p13.3	BB	BB	BB	BB	BB	BB	BB	rs323051	p13.3
AB	BB	BB	BB	AB	BB	BB	rs7195617	p13.3	NoCall	NoCall	AB	AA	AB	AB	AA	rs4627351	p13.3
AB	BB	BB	BB	AB	NoCall	BB	rs3830160	p13.3	BB	BB	AB	BB	AB	AB	BB	rs6600193	p13.3
AA	NoCall	AB	NoCall	NoCall	AB	AA	rs379387	p13.3	BB	BB	BB	NoCall	BB	NoCall	BB	rs13330069	p13.3
AA	NoCall	NoCall	NoCall	BB	NoCall	Exclude	rs4984666	p13.3	AA	AA	NoCall	NoCall	NoCall	NoCall	AA	rs10163266	p13.3
AA	AA	AA	AA	AB	AA	AA	rs763151	p13.3	BB	AB	AB	AA	AB	AB	Exclude	rs12926827	p13.3
BB	BB	BB	NoCall	NoCall	BB	BB	rs13335388	p13.3	BB	AB	AB	NoCall	AB	AB	BB	rs11642437	p13.3
AA	AA	AA	AA	AA	AA	AA	rs3743896	p13.3	NoCall	NoCall	NoCall	NoCall	NoCall	AB	AB	rs8060813	p13.3
AA	AA	AA	AA	AA	AA	AA	rs7195676	p13.3	AA	AB	AB	BB	NoCall	NoCall	Exclude	rs11644881	p13.3
AB	AB	AA	AA	AB	AA	AA	rs2178725	p13.3	AB	AB	AB	NoCall	BB	BB	BB	rs10500324	p13.3
AA	AA	NoCall	AA	AA	AA	AA	rs6600222	p13.3	BB	BB	NoCall	BB	BB	BB	BB	rs9941296	p13.3
AB	AB	BB	NoCall	NoCall	NoCall	BB	rs12921002	p13.3	AB	AA	AA	NoCall	AA	AB	AA	rs4785908	p13.3
AA	AA	NoCall	NoCall	NoCall	NoCall	AA	rs2071975	p13.3	AB	AA	AA	AA	AA	AB	AA	rs9925556	p13.3
AA	NoCall	AA	AA	AA	AA	AA	rs1054363	p13.3	AB	BB	BB	BB	BB	AB	BB	rs886953	p13.3
AA	AA	AB	AB	AA	NoCall	AA	rs743967	p13.3	AA	AA	AA	AA	AA	AB	AA	rs11076863	p13.3
NoCall	AA	NoCall	NoCall	AA	NoCall	AA	rs9934288	p13.3	AA	AA	AB	AA	AA	AA	AA	rs4785914	p13.3
AA	AA	AA	AA	AA	AA	AA	rs1045763	p13.3	AA	AA	AA	AA	NoCall	AA	AA	rs8053014	p13.3
BB	AB	BB	AB	AA	AB	Exclude	rs6597	p13.3	AA	AA	AB	AA	AA	AA	AA	rs2335461	p13.3
NoCall	AA	AA	AA	AA	AA	AA	rs1046112	p13.3	AA	AA	AA	AA	AB	NoCall	AA	rs2335462	p13.3
NoCall	AA	AA	AA	AA	AA	AA	rs7199133	p13.3	NoCall	BB	AA	AB	AB	AB	Exclude	rs8046063	p13.3
NoCall	BB	BB	BB	BB	BB	BB	rs6600231	p13.3	NoCall	NoCall	NoCall	NoCall	NoCall	BB	BB	rs4149797	p13.3
NoCall	AB	AB	AA	AA	AB	AA	rs11864516	p13.3	AA	AA	NoCall	NoCall	NoCall	NoCall	AA	rs7187296	p13.3
BB	BB	BB	BB	BB	BB	BB	rs17144313	p13.3	AB	AA	AB	AA	AA	AA	AA	rs2269911	p13.3
NoCall	AA	AA	AA	AA	NoCall	AA	rs11248951	p13.3	AB	NoCall	AB	NoCall	BB	BB	BB	rs7199221	p13.3
BB	BB	BB	BB	BB	BB	BB	rs11248952	p13.3	AB	AB	AB	NoCall	NoCall	NoCall	AB	rs1061019	p13.3
AA	AA	AA	NoCall	AA	NoCall	AA	rs7195271	p13.3	AA	AA	AB	AB	BB	AB	Exclude	rs1554999	p13.3
AA	AA	AA	AA	AA	NoCall	AA	rs1001366	p13.3	AB	BB	AB	BB	AB	AB	BB	rs2092015	p13.3
BB	NoCall	BB	BB	BB	NoCall	BB	rs4566169	p13.3	AB	BB	AB	BB	AB	AB	BB	rs4785926	p13.3
AA	AA	NoCall	AA	AA	AA	AA	rs13338480	p13.3	NoCall	AB	BB	AB	AB	AB	BB	rs909406	p13.3
AA	AA	BB	AB	AA	AA	Exclude	rs12923607	p13.3	AA	AB	AA	AB	AB	AB	AA	rs224177	p13.3
AA	AB	BB	BB	BB	BB	Exclude	rs17146072	p13.3	BB	BB	BB	BB	NoCall	BB	BB	rs7198126	p13.3
AA	AB	AB	BB	BB	BB	Exclude	rs3751666	p13.3	AB	NoCall	AB	BB	AB	AB	BB	rs2194337	p13.3
BB	BB	AB	BB	BB	BB	BB	rs6600238	p13.3	AB	BB	AB	BB	NoCall	AB	BB	rs1816116	p13.3
AB	BB	BB	BB	BB	NoCall	BB	rs535255	p13.3	NoCall	BB	BB	BB	BB	BB	BB	rs1816115	p13.3
AA	AB	AA	AA	AA	AA	AA	rs17135129	p13.3	AB	AA	AB	AA	AB	AB	AA	rs2741902	p13.3
AA	AA	AA	AA	NoCall	AA	AA	rs513000	p13.3	AA	AA	AB	AA	NoCall	AB	AA	rs1002965	p13.3
AB	AB	AB	AB	AA	AA	AA	rs11862437	p13.3	BB	BB	AB	BB	NoCall	AB	BB	rs1076573	p13.3
BB	AB	BB	AB	AA	AB	Exclude	rs213658	p13.3	BB	AB	NoCall	AB	AB	AB	BB	rs424165	p13.3
BB	AB	BB	AB	NoCall	AB	BB	rs12598318	p13.3	BB	BB	BB	BB	BB	BB	BB	rs9924261	p13.3
AB	AA	BB	BB	BB	AB	Exclude	rs12928460	p13.3	BB	BB	BB	BB	BB	BB	BB	rs9924466	p13.3
NoCall	BB	AA	AA	AA	AA	Exclude	rs909921	p13.3	AB	AA	AB	AA	AB	AA	AA	rs12917706	p13.3
NoCall	AA	AA	NoCall	NoCall	AA	AA	rs8044721	p13.3	NoCall	NoCall	NoCall	NoCall	BB	NoCall	BB	rs2075849	p13.3
NoCall	AA	AA	AA	AA	AA	AA	rs9933497	p13.3	BB	BB	BB	AB	AB	AB	BB	rs220382	p13.3
NoCall	NoCall	NoCall	NoCall	NoCall	AA	AA	rs3759937	p13.3	AA	AA	NoCall	AB	AB	AB	AA	rs12325636	p13.3
AB	AB	AA	AA	AA	AA	AA	rs4984834	p13.3	BB	BB	BB	AB	AB	AB	BB	rs17136317	p13.3
NoCall	AA	AB	AA	AB	AA	AA	rs742408	p13.3	AB	BB	BB	NoCall	AB	BB	BB	rs11645952	p13.3
AB	AB	AB	BB	AB	BB	BB	rs8045185	p13.3	AB	AA	AA	AB	BB	AB	Exclude	rs11861548	p13.3
BB	BB	NoCall	AA	AB	AA	Exclude	rs11645645	p13.3	AA	NoCall	AB	AA	AA	AA	AA	rs1234664	p13.3
AA	AA	AA	AA	AA	AB	AA	rs973675	p13.3	AA	NoCall	AB	AA	AA	NoCall	AA	rs1231089	p13.3
AB	AB	AA	NoCall	AA	AB	AA	rs973676	p13.3	AB	AB	AA	AB	BB	AB	Exclude	rs1968055	p13.3
AA	AA	BB	BB	AB	NoCall	Exclude	rs2667675	p13.3	BB	BB	AB	BB	BB	BB	BB	rs7200928	p13.3
NoCall	BB	BB	BB	BB	BB	BB	rs2235629	p13.3	BB	BB	AB	BB	BB	BB	BB	rs8060064	p13.3
BB	BB	BB	BB	BB	NoCall	BB	rs3180228	p13.3	AB	AB	AB	AB	NoCall	AB	AB	rs11643487	p13.3
AA	NoCall	NoCall	NoCall	AA	NoCall	AA	rs2745111	p13.3	AB	AB	AB	AB	BB	AB	BB	rs11648783	p13.3
NoCall	AA	AA	AA	NoCall	AA	AA	rs3784819	p13.3	AB	AB	AB	AB	AA	AB	AA	rs40633	p13.3
BB	NoCall	BB	BB	NoCall	BB	BB	rs3784820	p13.3	AB	AB	AB	AB	AA	AB	AA	rs27230	p13.3
AA	AA	AA	NoCall	NoCall	NoCall	AA	rs2667666	p13.3	AB	AB	AB	AB	AA	AB	AA	rs28603	p13.3
BB	BB	BB	NoCall	BB	BB	BB	rs2235643	p13.3	AB	AB	BB	AB	AA	AB	Exclude	rs3760083	p13.3
NoCall	NoCall	BB	NoCall	AB	BB	BB	rs2294624	p13.3	AA	AA	AB	AA	NoCall	AA	AA	rs37811	p13.3
NoCall	BB	BB	BB	NoCall	BB	BB	rs2076446	p13.3	AA	AA	AA	AA	AA	AA	AA	rs3901639	p13.3
AA	AA	AA	AA	AA	AA	AA	rs8049972	p13.3	NoCall	NoCall	AB	NoCall	NoCall	NoCall	AB	rs185149	p13.3
AB	BB	BB	BB	AB	AB	BB	rs2064289	p13.3	AB	AB	AB	AB	AA	AB	AA	rs250532	p13.3
BB	BB	BB	NoCall	BB	BB	BB	rs4558418	p13.3	AB	AB	AB	AB	AA	AB	AA	rs171634	p13.3
AA	AA	AA	AA	AB	AA	AA	rs2294623	p13.3	AB	AB	BB	AB	AA	AB	Exclude	rs3848356	p13.3
NoCall	BB	BB	BB	BB	BB	BB	rs8049566	p13.3	BB	BB	BB	BB	BB	BB	BB	rs2270496	p13.3
AA	AA	AA	AA	AA	AA	AA	rs11248883	p13.3	AB	AB	BB	AB	AA	AB	Exclude	rs11077337	p13.3
NoCall	BB	NoCall	BB	AB	NoCall	BB	rs10903010	p13.3	BB	BB	BB	BB	BB	BB	BB	rs17136419	p13.3
									NoCall	NoCall	NoCall	AB	NoCall	AB	AB	rs4786421	p13.3
									BB	AB	BB	NoCall	BB	NoCall	BB	rs2074523	p13.3

13.6 Appendix VI

All SNPs at the PBCRA 6q disease region:

III:34	IV:24	III:1	III:24	III:14	V:9	PBCRA AD	AV	LV	PV	V AD	PBCRA vs V	dbSNP RS ID	Cytoband
BB	AB	BB	AA	AB	AA	Exclude						rs7751232	q16.1
BB	AB	BB	AB	AB	AB	BB						rs2953370	q16.1
AA	AA	AA	BB	AB	BB	Exclude						rs9399724	q16.1
BB	AB	BB	AA	AB	AA	Exclude						rs6905412	q16.1
AA	AB	AB	BB	AB	BB	Exclude						rs6910024	q16.1
AA	AB	AB	BB	AB	BB	Exclude						rs6928585	q16.1
BB	AB	BB	BB	BB	BB	BB						rs4546494	q16.1
AA	AA	AB	BB	AB	AB	Exclude						rs9372382	q16.1
BB	BB	AB	AA	AB	AB	Exclude						rs7774322	q16.1
BB	BB	AB	BB	BB	BB	BB						rs4388292	q16.1
BB	BB	AB	BB	BB	BB	BB						rs4579361	q16.1
AA	AA	AA	AB	AA	AA	AA						rs4620131	q16.1
BB	AB	BB	AA	AB	AB	Exclude						rs9375155	q16.1
BB	BB	AA	BB	BB	BB	Exclude						rs3884624	q16.1
BB	AB	BB	AB	AB	AB	BB						rs4839952	q16.1
BB	AB	BB	AA	AB	AB	Exclude						rs9482760	q16.1
BB	BB	AB	BB	BB	BB	BB						rs7746024	q16.1
AA	AA	AB	AA	AA	AA	AA						rs4240564	q16.1
BB	BB	BB	BB	BB	AB	BB						rs12527609	q16.1
AA	AA	AB	AB	AB	BB	Exclude						rs2785895	q16.1
BB	BB	AB	AB	AB	AA	Exclude						rs2799655	q16.1
BB	BB	BB	BB	BB	BB	BB						rs494801	q16.1
BB	BB	BB	BB	BB	BB	BB						rs521715	q16.1
AA	AB	AB	AB	AB	AB	AA						rs1336969	q16.1
AA	AB	AA	AA	AA	AB	AA						rs579536	q16.1
BB	BB	BB	BB	BB	BB	BB						rs1590473	q16.1
AA	AB	AA	AA	AA	AA	AA						rs652797	q16.1
BB	BB	BB	BB	BB	BB	BB						rs1325075	q16.1
BB	BB	BB	BB	BB	BB	BB						rs17055922	q16.1
AB	AB	AB	AA	AB	AB	AA						rs1318073	q16.1
AB	AB	AB	BB	AB	AB	BB						rs4002794	q16.1
AB	AB	AB	AA	AB	AB	AA						rs9404179	q16.1
BB	AB	BB	BB	BB	BB	BB						rs11156184	q16.1
AA	AA	AA	AA	AA	AA	AA						rs4610576	q16.1
BB	BB	BB	BB	BB	BB	BB						rs4554330	q16.1
AB	AA	AA	BB	AB	AB	Exclude						rs9485739	q16.1
AB	AB	AA	BB	AB	BB	Exclude						rs9390936	q16.1
AA	AB	AA	AA	AA	AB	AA						rs12204966	q16.1
AB	BB	BB	AB	AB	BB	BB						rs9390961	q16.1
BB	BB	BB	BB	BB	BB	BB						rs4615399	q16.1
AA	AA	AA	AA	AA	AA	AA						rs4617001	q16.1
BB	BB	AB	BB	BB	BB	BB						rs4133134	q16.1
AA	AA	AA	AA	AA	AA	AA						rs4133133	q16.1
AA	AA	AA	AA	AA	AA	AA						rs4240600	q16.1
BB	BB	BB	BB	BB	BB	BB						rs6571117	q16.1
AA	AA	AA	AB	AA	AB	AA						rs12527888	q16.1
AB	AA	AA	AB	AB	AA	AA						rs9391137	q16.1
AA	AB	AB	AA	AB	AB	AA						rs6911299	q16.1
BB	BB	BB	BB	BB	BB	BB						rs17056495	q16.1
AB	AB	AA	BB	AB	BB	Exclude						rs4840253	q16.1
AB	BB	BB	AB	BB	BB	BB						rs2472923	q16.1
AB	AB	AB	BB	AB	BB	BB						rs12210146	q16.1
AB	AB	AB	AA	BB	AA	Exclude						rs9373833	q16.1
AB	AB	AB	AA	AB	AA	AA						rs11153023	q16.1
AB	AB	AB	AA	AB	AA	AA						rs2064947	q16.1
AA	AA	AA	AA	AA	AA	AA						rs9398152	q16.1
AB	AA	AB	BB	AA	AB	Exclude						rs211175	q16.1
AB	BB	AB	AA	BB	AB	Exclude						rs984520	q16.1
BB	BB	BB	BB	BB	BB	BB						rs11153105	q16.1
AA	AA	AA	AA	AA	AA	AA						rs4839854	q16.1
AB	AB	AB	AB	AB	AB	AB						rs17056960	q16.1
AA	AA	AA	AA	AA	AA	AA						rs9480968	q16.1
BB	BB	BB	BB	BB	BB	BB						rs213843	q16.1
AA	AA	AA	AB	AA	AA	AA						rs9487199	q16.1
AA	AA	AA	AB	AA	AA	AA						rs9320313	q16.1
AB	BB	BB	AB	BB	AB	BB						rs4839875	q16.1
BB	BB	BB	AB	BB	BB	BB						rs9487264	q16.1
AA	AA	AA	AA	AA	AA	AA						rs6568629	q16.1
AA	AB	AA	AA	AB	AA	AA						rs9400365	q16.1
AA	AB	AB	AB	AB	BB	Exclude						rs1206078	q16.1
AA	AA	AA	AA	AA	AA	AA						rs1206084	q16.1
BB	AB	AB	BB	AB	BB	BB						rs6917254	q16.1
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BB	BB	BB	BB	AB	AB	BB						rs6903354	q16.1
BB	BB	BB	BB	AB	AB	BB						rs6922974	q16.1
BB	BB	BB	BB	AB	AB	BB						rs6924307	q16.1
AA	AA	AA	AA	AB	AB	AA						rs17057182	q16.1
BB	BB	BB	BB	BB	BB	BB						rs7748086	q16.1
BB	BB	BB	AB	BB	BB	BB						rs946116	q16.1
AA	AB	AA	BB	AA	AB	Exclude						rs1766518	q16.1
AA	AB	AA	BB	AA	AB	Exclude						rs1737655	q16.1
AA	AA	AA	AA	AA	AA	AA						rs6917827	q16.1
AA	AB	AA	BB	AA	AB	Exclude						rs1884165	q16.1
AA	AB	AA	BB	AA	AB	Exclude						rs9384835	q16.1
BB	AB	BB	AB	BB	AB	BB						rs6929158	q16.1
AA	AA	AA	AA	AA	AB	AA						rs946120	q16.1
AA	AA	AA	AA	AA	AA	AA						rs7766733	q16.1
AA	AA	AA	AA	AA	AA	AA						rs7767908	q16.1
BB	BB	BB	BB	AB	AB	BB						rs1206149	q16.1
BB	BB	BB	BB	BB	BB	BB						rs1206125	q16.1
BB	AB	BB	BB	AB	AB	BB						rs1206129	q16.1
BB	BB	BB	BB	BB	BB	BB						rs9387143	q16.1
BB	BB	BB	BB	BB	BB	BB						rs9398363	q16.1
BB	BB	BB	BB	BB	BB	BB						rs7740726	q16.1
AA	AA	AA	AA	AA	AB	AA						rs7740887	q16.1
AA	AA	AA	AA	AA	AB	AA						rs9387218	q16.1
BB	BB	BB	BB	BB	AB	BB						rs4839709	q16.1
AA	AA	AA	AA	AA	AA	AA						rs9320500	q16.1

III:34	IV:24	III:1	III:24	III:14	V:9	PBCRA AD	AV	LV	PV	V AD	PBCRA vs V	dbSNP RS ID	Cytoband
AA	AA	AA	AA	AA	AA	AA						rs9320500	q16.1
AA	AA	AA	AA	AA	AA	AA						rs17806588	q16.1
BB	BB	BB	BB	BB	AB	BB						rs9320505	q16.1
BB	BB	BB	BB	BB	AB	BB						rs9387228	q16.1
BB	BB	BB	AB	BB	AB	BB						rs10499010	q16.1
BB	BB	BB	BB	BB	BB	BB						rs10499011	q16.1
AA	AA	AA	AB	AA	AB	AA						rs7771567	q16.1
AA	AA	AA	AA	AA	AA	AA						rs9488832	q16.1
BB	BB	BB	AB	BB	AB	BB						rs13210630	q16.1
AA	AB	AA	AA	AA	AA	AA						rs17057822	q16.1
BB	BB	BB	AB	BB	BB	BB						rs9401038	q16.1
AA	AA	AA	AB	AA	AB	AA						rs9374755	q16.1
AA	AA	AA	AB	AA	AB	AA						rs6937159	q16.1
BB	BB	BB	BB	BB	BB	BB						rs6936058	q16.1
BB	BB	BB	BB	BB	BB	BB						rs17057969	q16.1
AA	AA	AA	AA	AA	AA	AA						rs636598	q16.1
AB	BB	BB	BB	AB	BB	BB						rs636234	q16.1
AB	BB	BB	BB	BB	BB	BB						rs9481914	q16.1
AB	BB	BB	BB	BB	BB	BB						rs11968507	q16.1
AB	BB	BB	BB	BB	AB	BB						rs675974	q16.1
AA	AA	AA	AA	AA	AA	AA						rs9387768	q16.1
AA	AA	AA	AA	AA	AA	AA						rs6922758	q16.1
AA	AA	AA	AA	AA	AA	AA						rs6569153	q16.1
BB	BB	BB	BB	BB	BB	BB						rs6902595	q16.1
AA	AA	AA	AA	AA	AA	AA						rs11961238	q16.1
AB	AA	AA	AA	AA	AB	AA						rs6924808	q16.1
AB	AA	AA	AA	AA	AB	AA						rs9375023	q16.1
AB	AA	AA	AA	AA	AB	AA						rs9482159	q16.1
AB	AA	AA	AA	AA	AB	AA						rs4132635	q16.1
AA	AA	AA	AA	AA	AA	AA						rs9482263	q16.1
BB	BB	BB	BB	BB	BB	BB						rs17757989	q16.1
BB	BB	BB	BB	BB	BB	BB						rs2502933	q16.1
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BB	BB	BB	BB	BB	BB	BB						rs1466612	q16.2
AA	AB	AB	BB	AB	BB	Exclude						rs9320990	q16.2
BB	BB	BB	BB	BB	BB	BB						rs6932108	q16.2
AA	AA	AA	AA	AA	AA	AA						rs17058348	q16.2
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III:34	IV:24	III:1	III:24	III:14	V:9	PBCRA AD	AV	LV	PV	V AD	PBCRA vs V	dbSNP RS ID	Cytoband
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BB	BB	BB	BB	BB	BB	BB	AA	AA	AB	AA	Exclude	rs2173105	q16.2
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BB	BB	BB	BB	BB	BB	BB	AA	AA	AB	AA	Exclude	rs1496972	q16.2
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AB	AB	BB	BB	AB	BB	BB	AB	AA	AA	AA	AB	rs4320381	q16.3
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III:34	IV:24	III:1	III:24	III:14	V:9	PBCRA AD	AV	LV	PV	V AD	PBCRA vs V	dbSNP RS ID	Cytoband
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BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	rs1933990	q16.3
BB	BB	AB	BB	BB	AB	BB	BB	BB	BB	BB	BB	rs6906578	q16.3
BB	BB	AB	BB	BB	AB	BB	BB	BB	BB	BB	BB	rs12663577	q16.3
BB	BB	AB	BB	BB	AB	BB	BB	BB	BB	BB	BB	rs9499177	q16.3
BB	BB	AB	BB	BB	AB	BB	BB	BB	BB	BB	BB	rs9499178	q16.3
BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	BB	rs7451370	q16.3
BB	BB	AB	BB	BB	AB	BB	BB	AB	BB	BB	Exclude	rs10457109	q16.3
AB	AB	AB	AB	AA	AB	AA	AA	AA	AA	AA	AA	rs1996911	q16.3
AA	AA	AA	AA	AA	AA	AA	AA	NoCall	AA	AA	AA	rs9399768	q16.3
AA	AA	AA	AA	AA	AA	AA	AB	BB	BB	BB	BB	rs17063694	q16.3
AB	BB	BB	BB	BB	BB	BB	AB	AB	BB	BB	BB	rs1431222	q16.3
AB	AB	AB	BB	AB	AB	BB	AB	AB	AA	AA	AA	rs6918215	q16.3
AB	AB	AB	AA	AB	AB	AA	AA	AA	AA	AA	AA	rs9404274	q16.3
AA	AA	AA	AA	AA	AA	AA	AB	AB	AA	AA	AA	rs9485748	q16.3
AB	AB	AB	AA	AB	AB	AA	AA	NoCall	AB	AA	AA	rs9377412	q16.3
AA	AA	AA	AA	AA	AA	AA	AB	AB	AB	AB	BB	rs1323203	q16.3
AB	AB	BB	AB	BB	AB	BB	AA	AA	AA	AA	AA	rs9373672	q16.3
AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	rs2213828	q16.3
AA	AA	AA	AA	AA	AA	AA	AA	AB	AA	AA	AA	rs2157528	q16.3
AB	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	rs12192767	q16.3
AA	AA	AA	AA	AA	AA	AA	BB	BB	BB	BB	BB	rs2157527	q16.3
BB	BB	BB	BB	BB	BB	BB	AA	AA	AA	AA	AA	rs2205679	q16.3
AA	AA	AA	AA	AA	AA	AA	AA	AB	AA	AA	AA	rs7748098	q16.3
AB	AB	AA	AA	AA	AA	AA	AB	BB	BB	BB	BB	rs6571083	q16.3
BB	BB	AB	AB	AB	BB	BB	BB	AB	BB	BB	BB	rs9373682	q16.3
AB	BB	BB	BB	BB	BB	BB	AA	AB	AA	AA	AA	rs478938	q16.3
AB	AA	AB	AB	AA	AA	AA	AA	AB	AA	AA	AA	rs6571094	q16.3
AB	AA	AA	AA	AA	AA	AA	AB	BB	BB	BB	BB	rs2143795	q16.3
BB	BB	BB	BB	AB	BB	BB	AA	AA	AA	AA	AA	rs9390995	q16.3
AA	AA	AA	AA	AA	AA	AA	BB	BB	BB	BB	BB	rs17716862	q16.3
BB	BB	BB	BB	BB	BB	BB	AB	AB	AB	AB	AA	rs6902184	q16.3
AB	AA	AA	AB	AA	AA	AA	AA	AA	AA	AA	AA	rs9404372	q16.3
AA	AA	AA	AA	AA	AA	AA	AB	AB	AB	AB	AA	rs6936195	q16.3
AB	AA	AA	AB	AA	AA	AA	BB	BB	BB	BB	BB	rs9499431	q16.3
BB	BB	BB	BB	BB	BB	BB	AB	AB	AB	AB	AA	rs2207019	q16.3
AB	AA	AA	AB	AA	AA	AA	AB	BB	AB	BB	Exclude	rs2207021	q16.3
AB	AA	AB	AB	AB	AB	AA	AB	BB	AB	BB	BB	rs2503296	q16.3
AB	AB	BB	AB	AB	AB	BB	AB	AA	AB	AA	AA	rs2506743	q16.3
AA	AB	AB	AB	AA	AA	AA	AB	AA	AB	AA	AA	rs4947151	q16.3
AA	AA	AB	AB	AA	AA	AA	AB	BB	AB	BB	BB	rs12190784	q16.3
BB	AB	AB	AB	BB	BB	BB	AB	AA	AB	AA	AA	rs9391057	q16.3
AA	AB	AB	AB	AA	AA	AA						rs7759394	q16.3
AB	BB	AA	AA	AB	AB	Exclude						rs9499580	q16.3
BB	BB	AB	BB	BB	BB	BB						rs9377535	q16.3

III:34	IV:24	III:1	III:24	III:14	V:9	PBCRA AD	AV	LV	PV	V AD	PBCRA vs V	dbSNP RS ID	Cytoband
BB	BB	AB	BB	BB	BB	BB						rs9377535	q16.3
AB	BB	AB	AA	AB	AB	Exclude						rs7452930	q16.3
AB	AA	AB	BB	AB	AB	Exclude						rs9391078	q16.3
AB	BB	AB	AA	AB	AB	Exclude						rs7744535	q16.3
AB	BB	AB	AA	AB	AB	Exclude						rs9499614	q16.3
AB	AA	AB	BB	AB	AB	Exclude						rs9499615	q16.3
AB	AA	AB	BB	AB	AB	Exclude						rs9377543	q16.3
AA	AA	AA	AB	AA	AA	AA						rs2501182	q16.3
BB	BB	BB	BB	BB	BB	BB						rs2506732	q16.3
AB	AA	AB	BB	AB	AB	Exclude						rs9373718	q16.3
AB	AB	AB	AB	AB	AB	AB						rs12206299	q16.3
BB	BB	BB	BB	BB	BB	BB						rs7765474	q16.3
AB	AA	BB	AB	AB	AB	Exclude						rs4476862	q16.3
BB	BB	BB	BB	BB	BB	BB						rs9322774	q16.3
AB	AB	AA	AA	AA	AA	AA						rs3922556	q16.3
AA	AB	AB	AB	AB	AB	AA						rs4484555	q16.3
AB	AB	AA	AA	AA	AA	AA						rs4144638	q16.3
AB	AB	BB	BB	BB	BB	BB						rs9322783	q16.3
BB	AB	AB	AB	AB	AB	BB						rs859966	q16.3
BB	BB	BB	BB	BB	BB	BB						rs9485928	q16.3
AB	BB	AA	AB	AA	AA	Exclude						rs9377620	q16.3
AB	BB	AB	AB	AA	AA	Exclude						rs1365686	q16.3

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