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Genetic factors of age-related macular degeneration

Jingsheng Tuo, Christine M. Bojanowski, and Chi-Chao Chan*

Laboratory of Immunology, Immunopathology Section, National Eye Institute, National Institutes of Health, Bldg. 10, Rm. 10N103, 10 Center Drive, Bethesda, MD 20892-1857, USA

Abstract

Age-related macular degeneration (AMD) is a leading cause of blindness in the United States and developed countries. Although the etiology and pathogenesis of AMD remain unknown, a complex interaction of genetic and environmental factors is thought to exist. The incidence and progression of all of the features of AMD are known to increase significantly with age. The tendency for familial aggregation and the findings of gene variation association studies implicate a significant genetic component in the development of AMD. This review summarizes in detail the AMD-related genes identified by studies on genetically engineered and spontaneously gene-mutated (naturally mutated) animals, AMD chromosomal loci identified by linkage studies, AMD-related genes identified through studies of monogenic degenerative retinal diseases, and AMD-related gene variation identified by association studies.

1. Introduction

Age-related macular degeneration (AMD) is a chronic and progressive degeneration of photoreceptors, the underlying retinal pigment epithelium (RPE), Bruch's membrane and possibly the choriocapillaries in the macula (Fine et al., 2000; Hageman et al., 2001; Luty et al., 1999). The macula is a highly specific region, 6 mm in diameter, located in the central retina temporally to the optic disc. At the center of the macula lies the fovea, 0.35 mm in diameter, which contains a population of photoreceptor cells responsible for reading and color vision. Clinical and pathological features of AMD include alteration of Bruch's membrane and RPE cells, drusen formation, hypo- and/or hyperpigmentation, loss of photoreceptors, choroidal neovascularization, and subretinal fibrous and/or neovascular tissue in the macula. These alterations result in the loss of central visual acuity (Ambati et al., 2003a). Although laser photocoagulation targeting the choroidal feeding vessels, photodynamic therapy, surgical macular translocation, and antiangiogenesis agents are reported to reduce the risk of moderate to severe vision loss in some patients with the neovascular, or wet, form of the disease, there is no proven treatment of advanced AMD to date (Ciardella et al., 2002; Fujii et al., 2003; Lim, 2002; McBee et al., 2003; Rechtman et al., 2002).

AMD is the leading cause of visual impairment and blindness in the United States and the developed world among people 65 years and older (la Cour et al., 2002). AMD prevalence was observed in 9% of the population from ages 52–85 years old in the Framingham study and in 33% of autopsied eyes obtained after death from people older than 65 years (Kini et al., 1978; Kornzweig, 1965). Roughly 30% of the human population 75 years or older has some degree of AMD. As the average life span of humans continues to increase, particularly in the developed countries, the incidence of AMD is expected to nearly double within the next 25 years (Smith et al., 2001).

*Corresponding author. Tel.: +1-301-496-0417; fax: +1-301-402-8664. E-mail address: chanc@nei.nih.gov (C.-C. Chan).

Currently, the etiology of AMD remains elusive. In addition to age, recognized risk factors include cigarette smoking and diet (Age-Related Eye Disease Study Research Group, 2000, 2001; Husain et al., 2002; Hyman and Neborsky, 2002; Seddon et al., 2001). RPE dysfunction mediated by reactive oxygen intermediates has also been suggested as a possible cause of AMD (Beatty et al., 2000). Due to the importance of antioxidants in scavenging free radicals, many investigators have speculated that dietary antioxidants and mineral cofactors confer protection against AMD (Frank, 1998; Frank et al., 1999). Oxidative stress may promote neovascularization via deposition of extracellular matrix along Bruch's membrane and increased levels of the angiogenic factors in RPE cells (Mousa et al., 1999). The results of the age-related eye disease study (AREDS, 2001) suggest a moderate benefit of antioxidant, vitamin and mineral supplementation in curbing the progression of neovascular AMD. Different opinions are held concerning the association between AMD and hypertension (Delcourt et al., 2001; Hyman and Neborsky, 2002; Snow and Seddon, 1999). Among all identified potential environmental factors, only age, diet and smoking are agreed upon by all to increase the risk of AMD.

The pathogenesis responsible for AMD involves environmental factors and varying susceptibilities to these external factors based upon different genetic backgrounds (Guymer, 2001). Ethnicity, for example, is a component of genetic background. AMD is more prevalent in people with less pigmentation, although little difference in the occurrences of smaller-size drusen in individuals with light and dark skin has been reported (Age-Related Eye Disease Study Research Group, 2000, 2001; Friedman et al., 1999). A possible explanation for this observed trend suggests an enhanced protective role for the melanin in RPE cells and choroidal melanocytes. There has been compelling documented evidence that heredity plays a role in AMD (Yoshida et al., 2000). The tendency for familial aggregation in AMD cases further points to a genetic background for the disease. Approximately 20% of AMD patients have a positive family history (de Jong et al., 1997; Klaver et al., 1998b; Meyers et al., 1995). Furthermore, there is higher prevalence of AMD among monozygotic twins as compared to their spouses and first-degree relatives (Gottfredsdottir et al., 1999; Klaver et al., 1998b). Recently, the potential role of genetic variation in AMD development has gained attention. Gene variation has been reported in association with various other human age-related diseases such as cancer and the cardiovascular diseases (Brennan, 2002; Gulcher et al., 2001; Halushka et al., 1999). A few small studies have also demonstrated the association between AMD and various gene polymorphisms (Husain et al., 2002; Schmidt et al., 2000). This review will discuss in detail the currently known genetic risk factors associated with AMD.

2. AMD-related genes identified by genetically engineered or spontaneously gene-mutated (naturally mutated) animals

Characterization of animals with retinal degeneration due to genetic engineering or spontaneous gene mutations is an important approach for obtaining information on critical retinal degeneration pathways. Knowledge of the genetic homologies and similar phenotypes between mice, rats, and humans could lead to the identification of relevant human genes.

2.1. Genetically engineered animal models

AMD-like clinical and pathological features have been induced in genetically modified animals. A series of retinal degeneration animal models have been established based on the known retinal degeneration-related genes identified through studying monogenetic retinal diseases and gene variation association studies as discussed below. Various clinical signs of retinal degeneration are mimicked in *ABCA4* (a photoreceptor-specific ATP-binding cassette transport gene, alternatively named *ABCR*) knockout mice (Mata et al., 2001; Weng et al., 1999); *Apolipoprotein E* (*ApoE*), a ligand for the low-density lipoprotein (LDL) receptor

family) knockout mice (Dithmar et al., 2000); Apo(*)E3-Leiden mice that carry a dysfunctional form of human ApoE- ϵ 3 discovered in a family from the Dutch town Leiden (Kliffen et al., 2000); and knock-in mice that carry a disease-related Ser156Cys mutation in the *tissue inhibitor of metalloproteinases-3 (TIMP3)* gene (Weber et al., 2002).

Through genetically engineered animal studies alone, several retinal degeneration-related genes have been identified. The phenotype of *RPE65* knockout mice indicates a role for rhodopsin in retinal degeneration. The *RPE65* gene has been refined to human chromosome 1p1 and mouse chromosome 3. *RPE65* is an RPE-specific 65-kDa protein localized to the microsomal membranes of vertebrate RPE. *RPE65* functions in the isomerization of 11-*trans* to 11-*cis* retinol and partly in RPE rhodopsin regeneration (Hamel et al., 1993,1994). The *RPE65* knockout in mice confers a sensitivity to light damage which mimics certain aspects of retinal degeneration (Gouras et al., 2002;Katz and Redmond, 2001;Rohrer et al., 2003;Wenzel et al., 2003). Spontaneous progressive photoreceptor degeneration was also found in *RPE65* knockout mice (Gouras et al., 2002). However, *RPE65* is unlikely relevant to AMD.

Another animal model for retinal degeneration is based on the *mcd/mcd* transgenic mouse (Rakoczy et al., 2002). Cathepsin D (CatD) is an important and widely expressed aspartic protease responsible for the digestion of phagocytosed opsin photoreceptor outer segments by the RPE. RPE cells have been shown to express high levels of CatD. Changes in cathepsin's lysosomal activity have been associated with many diseases. CatD is first produced in a pre-procathepsin form and then subjected to a series of cleavage and activation steps. The *mcd/mcd* transgenic mouse expresses a mutated form of *cathepsin D (CatD)* that mimics human *CatD* lacking Glu44p and Gly1 cleavage sites (Rakoczy et al., 2002). Circulating levels of CatD are not affected by these mutations and remain high. Improper cleavage, however, does render circulating CatD enzymatically inactive. These inactive forms are thought to impair rod outer-segment degradation, which eventually leads to the development and accumulation of hyalinized drusen (Rakoczy et al., 1996,1999). In old age, the *mcd/mcd* mice exhibit most features of human AMD such as RPE pigmentary changes, attenuation, hyper- and hypoplasia, proliferation and atrophy of RPE cells, accumulation of basal laminar and linear deposits, and decreased retinal sensitivity. Under the light microscope, geographic atrophy appears in the advanced stage (Rakoczy et al., 1996,2002).

Mouse knockouts of the C-C chemokine Ccl2 (MCP-1) or its cognate receptor-2 (Ccr2) spontaneously develop various cardinal features of AMD in their senescence stage. These observations suggest that normal Ccl2/Ccr2 function confers some degree of protection against AMD (Ambati et al., 2003b). Ccl2 and Ccr2 are involved in monocyte and macrophage chemoattraction. Ccl2 and Ccr2 also possibly play roles in degrading the deposits accumulated over age in Bruch's membrane. Different from other AMD animal models, in which only the dry type or wet type of AMD is mimicked by either drusen formation or neovascularization, respectively; Ccl2 and Ccr2 knockouts develop both dry and wet AMD types with the wet type as the advanced stage. The pathological changes in these aged mice include photoreceptor atrophy, choroidal neovascularization, accumulation of lipofuscin in the RPE, and the formation of drusen beneath the RPE. As in humans, complement and IgG deposition in the choroid accompanies senescence in these animal models. RPE or choroidal endothelial production of Ccl2 induced by complement C5a and IgG may mediate choroidal macrophage infiltration in the aged wild-type choroid. Therefore, impaired macrophage recruitment possibly results in C5a and IgG accumulation, which in turn induces vascular endothelial growth factor (VEGF) production by the RPE. VEGF is a possible mediator of choroidal neovascularization development. The Ccl2/ Ccr3 model implicates macrophage dysfunction in the pathogenesis of AMD and may be a useful platform in validating therapies (Ambati et al., 2003b).

2.2. Spontaneously gene-mutated (naturally mutated) animal models

Mouse strains with spontaneous retinal degenerations have been used for many years to identify genes that cause retinal degeneration. Many of these animal models come from screening genetically independent mouse strains and stocks using indirect ophthalmoscopy and electroretinography (Chang et al., 1993,1994;Heckenlively et al., 1989). These mice develop photoreceptor dysfunction leading to the death of both rod and cone photoreceptors. The mechanism behind photoreceptor cell death, which mimics the dry form of AMD, is thought to be the process of apoptosis (Chader, 2002).

The retinal degeneration 6(*rd6*) mouse, from the C3HfB/GaCas1b strain, is one animal model identified by such genomic screenings (Kameya et al., 2002). The inheritance pattern of *rd6* is autosomal recessive. Fundus examination of *rd6* homozygous mice shows distribution of discrete dots across the retina. Mice homozygous for the *rd6* mutation exhibit retinal dysfunction and a slow progressive photoreceptor degeneration (Hawes et al., 2000). *Mfrp* within the *rd6* region was identified as responsible for the photoreceptor degeneration in these *rd6* mice by positional cloning. Other animal models in the *rd* series have been intensively reviewed by Chang et al. (2002).

Bst⁺ is a spontaneous, autosomal, semi-dominant mouse mutation in the Belly spot and tail (*Bst*) gene mapped to mouse chromosome 16, 1.9±1.1 cM from *D16Mit168* (Rice et al., 1997). In early onset, the main retinal features of *Bst*⁺ mice are summarized as defective development. In mice older than 7 months of age, choroidal neovascularization is seen to extend into the outer portion of the sensory retina. This neovascularization pattern is similar to that observed in the advanced stage of human wet-type AMD. Cloning of the *Bst* gene and the identification of the homologous human gene may be useful in uncovering this gene's role in choroidal neovascularization (Smith et al., 2000).

The RD locus was first identified by retinal degeneration linkage studies carried out in mice (Sidman and Green, 1965). The *RDS* gene was originally identified in a strain of mice with a form of photoreceptor degeneration termed "retinal degeneration, slow" (*rds*) (Demant et al., 1979;van Nie et al., 1978). The *rds* mouse, which, in genotype, is homozygous for a null mutation in *peripherin* / *RDS*, completely fails to develop photoreceptor discs and outer segments, and exhibits down-regulation of rod opsin expression and apoptotic loss of photoreceptor cells (Kohl et al., 1998). *Peripherin/RDS* mutations in codons 169, 172, and 195 have been identified in autosomal dominant macular dystrophy (Downes et al., 1999;Ekstrom et al., 1998;Lith-Verhoeven et al., 2003b;Nakazawa et al., 1995;Payne et al., 1998;Piguet et al., 1996;Wells et al., 1993;Yanagihashi et al., 2003). Recently, Khani et al. (2003) reported that a *RDS* mutation in codon 141 is associated with an unusual AMD-like late-onset maculopathy.

3.AMD chromosomal loci identified by linkage studies

A genome-wide scan of extended families with AMD is one of the approaches taken to locate candidate AMD loci. The total relative likelihood for a possible linkage relationship among selected loci is expressed by a lod, an acronym for "logarithm of odds", score. A lod score of 3 is generally considered to be an acceptable criterion for demonstration of loci linkage. A threshold of lod = 2 is often used as a criterion for suggestive linkage (Majewski et al., 2003). The Hlod parameter, which takes into account both lod score likelihood estimations and heterogeneity, was a necessary addition to linkage studies of complex diseases such as AMD. Lander and Kruglyak (1995) have recommended that a lod score of 3.3 be considered a reasonable criterion for linkage in studies using lod score analysis in humans. Slightly higher lod scores, for example, 3.6–3.8, they suggest, might be appropriate for allele sharing methods. Majewski et al. (2003) recently reported multiple loci in linkage by a genome-wide scan in 70

families, ranging from small nuclear families to extended multigenerational pedigrees. This study included a total of 344 AMD cases and 217 unaffected members. Findings in this study confirmed the linkage previously identified at chromosome 1q25–31 in a single large family (Majewski et al., 2003). This locus was also previously reported by other investigators (Klein et al., 1998; Schick et al., 2003; Weeks et al., 2001). However, the locus was defined at D1S518 by Majewski et al. (2003), between markers D1S466 and D1S413 by Klein et al. (1998), and between markers D1S1660 and D1S1647 by Schick et al. (2003).

In the same study, Majewski et al. (2003) also achieved a Hlod score of 3.06 with marker D10S123 on chromosome 10q26. This locus has also been reported elsewhere (Weeks et al., 2001; Kenealy et al., 2004). Through the use of correlation analysis, a statistically significant correlation was established between lod scores at 10q26 and 3p13, another AMD locus identified in this study. Loci at 4q32 (D4S2368) and 3p13 (D3S1304/D3S4545) have been linked to the predominantly dry-AMD phenotype in a subgroup of families with Hlod scores of 2.66 and 2.19, respectively (Majewski et al., 2003). D9S934 at chromosome 9q33 has been linked to AMD with a lod score of 2.01 in 21 families (Majewski et al., 2003).

In addition to the loci at chromosome positions 1q31 and 10q26, a study of 860 affected individuals from 391 families (452 sib pairs) reported linkage to markers on chromosome 17q25 (Weeks et al., 2001). Using their most stringent diagnostic model, Weeks et al. (2001) found the 17q25 Hlod score at D17S928 was 3.16. This locus has also been reported elsewhere (Schick et al., 2003). Positive lod scores of 3.59 and 3.1 were reported for D6S1644 and D6S249, respectively, at chromosome 6q14 (Kniazeva et al., 2000). Seddon et al. (2003) reported the linkage of AMD to D22S1045 at chromosome 22q12, and D2S1391 and D2S1384 at chromosome 2q31–32. Table 1 summarizes the chromosomal loci with lod scores greater than 2 identified in linkage studies with AMD.

Most recently, Iyengar et al. (2004) reported a novel major AMD locus on chromosome 15q21 (GATA50C03/D15S659) with $P = 2 \times 10^{-7}$ by dissecting genome-wide scan data of extended families and using a quantitative trait linkage approach. In this study, AMD was graded on a 16-level quantitative scale for severity based on drusen size, type, and area; pigmentary abnormalities; geographic atrophy; and signs of exudative macular degeneration. This quantitative scale thus provides increased power in detecting linkage signals as compared to the study carried out by Schick et al. (2003) using the assignment of binary affection status based on clinical criteria or threshold value from the same data pool.

AMD genes can also be identified following further paring down and refining of linked AMD loci. Schultz et al. (2003b) recently reported an AMD gene, *hemicentin-1*, after further mapping of the *ARMD1* region in 1q25–31. This region was previously discovered in a genome-wide scan (Klein et al., 1998). There are roughly 50 genes within the *ARMD1* region. Mutation screening targets were prioritized based on their expression in the retina, their similarity to genes known to cause retinal disorders, and whether or not they encode extracellular matrix proteins or proteins potentially related to drusen formation. A total of 20 genes in the refined *ARMD1* region were screened. Through these screens, an A16263G variation in exon 104 of *hemicentin-1* was found. This site is in conjunction with the disease haplotype although it was not found in conjunction with any of the other 17 haplotypes reported across the *ARMD1* region in the pedigree studied (Schultz et al., 2003b). None of the 11 family members who lacked the disease haplotype carried the A16263G variation, an observation which further strengthens the significance of this finding.

The A16263G variation causes a non-synonymous substitution of arginine for glutamine at amino acid position 5345 (Gln5345Arg). Alignment of the amino acid sequence of a portion of *hemicentin-1* exon 104 in humans shows that Gln5345 is conserved in seven mammalian

species as well as in chickens. Little is known concerning the function of hemicentin-1. The protein sequence of hemicentin-1 is similar to that of hemicentin (Vogel and Hedgecock, 2001), a conserved extracellular matrix protein belonging to the immunoglobulin superfamily in *Caenorhabditis elegans*. Hemicentin functions as an organizer of epithelial and other cell attachments into oriented line-shaped junctions. Mutations in *him-4*, the gene which codes hemicentin, cause tissue fragility, defective cell migration and chromosome instability in *C. elegans*. The predicted protein sequence of *hemicentin-1* resembles *EFEMP1* (EFG-containing fibulin-like extracellular matrix protein-1), a causative gene in autosomal dominant radial drusen development (see below). Both *hemicentin-1* and *EFEMP1* encode extracellular matrix proteins that contain a series of calcium-binding epidermal growth factor-like domains followed by an unusual carboxy terminal EGF-like domain with eight cysteines.

Iyengar et al (2004) followed linkage analysis of the extended families-based AMD cases with testing the sequence variation of *hemicentin-1* in 1q31 linked families. Instead of finding any variation in exon 104 of *hemicentin-1*, four *hemicentin-1* SNPs (see Section 5), rs1475113 in intron 4, rs743137 in intron 36, hCV625089 in intron 86, and rs680638 in intron 105, were found to possibly be AMD related. Inclusion of these SNPs as markers in the linkage model has contributed significantly to the overall evidence for linkage at 1q31.

4. AMD-related genes identified by studies of monogenic degenerative retinal diseases

There are many limitations in causative gene mapping through linkage studies in late-onset diseases such as AMD. First, many individuals in the affected families are young and have not yet reached the age of disease onset (which, in the case of AMD, is over the age of 50 years); second, it is likely that many older family members have passed away making informative individuals from multiple generations rarely available; and third, the heterogeneity of AMD requires the estimation of multiple genetic risks as this disease is believed to result from mutations in two or more different and unrelated genes rather than from single gene disruption. Furthermore, the variable presentation of AMD leads to difficulty and imprecision in the classification of the affected individuals (Seddon et al., 2003). For these reasons, the study of other hereditary monogenic macular and retinal dystrophies that share clinical and pathological similarities with AMD may offer some insightful clues in the search to identify AMD-related genes.

4.1. Stargardt macular dystrophy (STGD)

Various studies have reported *ABCA4* in 1p21 as a causative gene of Stargardt macular dystrophy (STGD), a blinding autosomal recessive retinal dystrophic disease that affects children and teenagers (Kaplan et al., 1993). STGD is characterized by delayed dark adaptation and accelerated deposition of lipofuscin in the RPE (Allik-mets et al., 1997b; Birnback et al., 1994). STGD is of high heterogeneity and shares many features with AMD. The *ABCA4* gene encodes the rim protein (RmP), an integral membrane glycoprotein localized to the rims of photoreceptor outer-segment discs (Azarian and Travis, 1997). RmP belongs to the ABC transporter superfamily and possibly functions as a flippase for *N*-retinylidene-phosphatidylethanolamine, the normally occurring Schiff-base conjugate of phosphatidylethanolamine with all-*trans*-RAL (Mata et al., 2001). Deposition of the lipofuscin fluorophore *N*-retinylidene-*N*-retinylethanolamine (A2E) is seen in the STGD RPE layer (Weng et al., 1999). Accumulation of lipofuscin in RPE cells is observed in several forms of macular degeneration including AMD (Kliffen et al., 1997; Weng et al., 1999). Slow accumulation of lipofuscin is also seen during normal aging (Kennedy et al., 1995). RmP may prevent A2E deposition in RPE cells by eliminating A2E precursors from the photoreceptors. The association studies on sequence variants of *ABCA4* and AMD are discussed below.

PROML1 has been identified as another causative gene for STGD (Maw et al., 2000). The gene *PROML1* in 4p15 encodes human prominin (mouse)-like 1 (previously known as AC133 antigen), which belongs to the prominin family of five-transmembrane domain proteins. *PROML1* is expressed in retinoblastoma cell lines and adult retina. The product of the mouse orthologue (prom) is concentrated in membrane evaginations at the base of the rod photoreceptor outer segments (Maw et al., 2000). Screening of a STGD4 pedigree revealed that affected individuals were homozygous for a nucleotide 1878 deletion in *PROML1*. This alteration is predicted to result in a frameshift at codon 614 that causes the premature termination of translation. Functional studies in transfected CHO cells have demonstrated that the truncated prominin protein fails to reach the cell surface, suggesting that the loss of prominin may lead to retinal degeneration via the impaired generation of evaginations or conversion to outer-segment disks (Maw et al., 2000).

4.2. Sorsby fundus dystrophy (SFD)

Sorsby fundus dystrophy (SFD) is an autosomal dominant disease clinically characterized by choroidal neovascularization and irregularities in the extracellular matrix of Bruch's membrane. SFD leads to an onset of night blindness in the third decade of life and a loss of central vision from macular atrophy by the fifth (Gregory-Evans, 2000; Weber et al., 1994a). The SFD locus was mapped to 22q13-qter, containing the gene for *TIMP3*. *TIMP3* is known to play a pivotal role in the regulation of the matrix metalloproteinases, such as the collagenases and gelatinases. *TIMP3* thereby determines the extent of matrix degradation during normal tissue remodeling and regulates the homeostasis of the extracellular matrix (Weber et al., 1994a).

Point mutations in *TIMP3* have been reported in affected members of two SFD pedigrees (Weber et al., 1994a). These mutations are predicted to disrupt the tertiary structure and consequently the functional properties of the mature protein. Mutations in *TIMP3*, including Ser181Cys (Carrero-Valenzuela et al., 1996; Weber et al., 1994a), Ser156Cys (Felbor et al., 1996; Soboleva et al., 2003), and Tyr172Cys (Jacobson et al., 2002), occur primarily in the C-terminal region of exon 5. The majority of the reported mutations in *TIMP3* lead to a replacement of reference residue by cysteine residues. This substitution results in aberrant disulfide bond formation and an abnormal tertiary protein structure. These changes most likely alter *TIMP3*-mediated extracellular matrix turnover thereby leading to the thickening of Bruch's membrane and the widespread accumulation of abnormal material beneath the RPE observed histologically (Capon et al., 1989; Chong et al., 2000).

The *TIMP3* protein plays an important role in the regulation of choroidal neovascularization, a prominent feature in SFD as well as in the advanced stages of AMD (Anand-Apte et al., 1997). In vitro and in vivo vascular endothelial migration assays indicate that *TIMP-3* inhibits chemotaxis of vascular endothelial cells toward VEGF and basic fibroblast growth factor (bFGF). In vitro, collagen gel invasion and capillary morphogenesis is inhibited. In vivo, bFGF-induced angiogenesis is reduced in the chorioallantoic membrane assays. Thus, *TIMP3* has the potential to inhibit angiogenesis, most likely by blocking VEGF-2 receptors (Anand-Apte et al., 1997). In addition, Majid et al. (2002) have demonstrated that mutant *TIMP3* can induce apoptosis of RPE cells. This finding suggests that apoptosis may be the final pathway for cell death in SFD.

4.3. Best disease

VMD2 (alternatively referred to as the *Bestrophin* gene) has been identified as the causal gene of dominant juvenile onset vitelliform macular dystrophy, commonly known as Best disease. Best disease affects the central retina and is characterized clinically by the classical abnormal electrooculogram and the presence of round or oval yellow subretinal macular deposits. The

yellow material gradually reabsorbs over time, leaving an area of RPE atrophy and often subretinal fibrosis (Kramer et al., 2003; Michaelides et al., 2003; Petrukhin et al., 1998; Weber et al., 1994b). Occasionally, at the time of disintegration, there may be hemorrhage into the retina. Histopathology of autopsied eyes from a Best disease patient showed accumulation of lipofuscin throughout the RPE, similar to AMD (Frangieh et al., 1982). This disease exhibits extremely variable expressivity.

Most affected individuals carry mutations in the *VMD2* gene (Kramer et al., 2003). The *VMD2* gene, containing 11 exons spanning 14.1 kb of genomic DNA, has been localized to 11q13.2–13.3 (Weber et al., 1994b). The gene encodes a 585-amino-acid protein known as bestrophin, which is selectively expressed in the RPE of the eye (Marquardt et al., 1998; Petrukhin et al., 1998). Bestrophin has sequence homology with the RFP protein family. These proteins have a conserved sequence of 350–400 amino acids and are found in organisms as diverse as the nematode, the fruit fly, and the mouse. The function of this family of proteins is currently unknown although bestrophin has been characterized as a possible chloride channel (Marquardt et al., 1998; Petrukhin et al., 1998). Analysis of 259 patients with AMD has shown no significant role of *bestrophin* mutation in AMD (Allikmets et al., 1999). This is confirmed in another independent study of 85 non-familial AMD patients (Akimoto et al., 2001).

4.4. Malattia leventinese and Doyme honeycomb retinal dystrophy

The disruption of *EFEMP1* at 2p16–21 has been linked with *Malattia leventinese*, a dominant macular dystrophy, and Doyme honeycomb retinal dystrophy. Both of these disorders are characterized by confluent drusen accumulation beneath the RPE, an early hallmark of AMD (Heon et al., 1996; Stone et al., 1999). *EFEMP1* is expressed in the tissues closest to the site of drusen formation (RPE, retina, and choroid) and encodes a protein homologous to a family of extracellular matrix glycoproteins known as the fibulins (Tran et al., 1997). *EFEMP1* was originally isolated as a cDNA sequence (formerly S1-5 and FBNL) that was over-expressed in human fibroblasts obtained from a patient with Werner syndrome, a genetic disease characterized by accelerated ageing (Lecka-Czernik et al., 1995). It has been reported that abnormal accumulation of EFEMP1 in the eyes may accelerate drusen formation and cellular degeneration (Marmorstein et al., 2002). EFEMP1 is an extracellular matrix protein. The high likelihood of the interaction between extracellular matrix proteins, such as adhesion molecules, collagens, elastins, fibronectins, laminins, tenascins, hemicentins and vitronectins, suggests an entire group of genes as possible candidates for involvement in drusen formation (Stone et al., 1999).

4.5. Pattern dystrophies

Disruptions in the *peripherin/RDS* gene on chromosome 6p21 have been identified in patients with pattern dystrophies characterized by a bilateral accumulation of lipofuscin-like material at the level of the RPE (Nichols et al., 1993a,b). These disruptions include the missense mutations Gly167Asp, Pro210Arg, Pro216Ser, and Cys213Tyr, as well as the protein truncating mutations Gln331 stop, a 4-base pair (bp) deletion at codon 140, a 2-bp frameshift deletion at codon 290, a 2-bp frameshift deletion at codons 299/300, and a large deletion that removes exons 2 and 3 of the *peripherin/RDS* gene (Lith-Verhoeven et al., 2003a). The peripherin/RDS protein is a membrane-associated glycoprotein restricted to photoreceptor outer-segment discs in a complex with ROM1. This protein may function as an adhesion molecule involved in the stabilization and maintenance of a compact arrangement of outer-segment discs (Travis et al., 1991). Peripherin has also been shown to interact with the GARP domain (glutamic acid- and proline-rich region) of the beta-subunit of rod cGMP-gated channels in a complex including the Na/Ca–K exchanger (Poetsch et al., 2001). This interaction may have a role in anchoring the channel–exchanger complex in the rod outer-segment plasma membrane. A recent study has shown no association of *peripherin/RDS* with Butterfly-shaped

macular dystrophy, a type of pattern dystrophy, underscoring the genetic heterogeneity characteristic of these diseases (Lith-Verhoeven et al., 2003a).

4.6. Late-onset retinal degeneration (L-ORD)

CTRP5 has recently been reported as the causal gene of late-onset retinal degeneration (L-ORD), an autosomal dominant disorder with striking clinical and pathological similarity to AMD (Hayward et al., 2003). Clinically, L-ORD is characterized by onset in the fifth to sixth decade of life with night blindness as the main clinical feature. Pathological features include thick punctuate yellow-white deposit between the basal lamina of the RPE and Bruch's membrane, progression to severe central and peripheral degeneration, and choroidal neovascularization and chorioretinal atrophy (Kuntz et al., 1996; Milam et al., 2000). L-ORD is distinct from AMD only in its Mendelian inheritance pattern and the pathological tendency towards a more extensive distribution of sub-RPE deposit and atrophy.

A 10 cM genome scan in an extended L-ORD family enabled Hayward et al. (2003) to identify linkage to a 15 cM interval in 11q23.3, flanked by D11S4127 and D11S4151, with a maximum lod of 6.77. Candidate gene analysis discovered a mutation in the *CTRP5* gene which codes a short-chain collagen. The mutation of a highly conserved serine to arginine (Ser163Arg) in the gC1q domain was found in 7/14 L-ORD families and 0/1000 control individuals, suggesting that this mutation is a L-ORD-causing founder mutation. *CTRP5* is secreted by the RPE and is a potential constituent of Bruch's membrane. This protein most likely functions in the facilitation of basal RPE adhesion to Bruch's membrane by forming an extracellular hexagonal lattice. The Ser163Arg mutation likely impairs this adhesion process, thereby leading to the build-up of extracellular deposit. Another possible consequence of this mutation may be the formation of an abnormally high molecular weight aggregate, which in turn may alter the higher-order structure and consequently the interactions of the molecule. The precise role of *CTRP5* remains to be established; however, the presence of a common founder mutation in L-ORD suggests that this protein exerts an important influence on age-related build-up of extracellular deposits, as seen in both L-ORD and AMD (Apfelstedt-Sylla et al., 1995; Jacobson et al., 2001; Kuntz et al., 1996; Milam et al., 2000). These results further imply that impaired adhesion between RPE and Bruch's membrane may be one of the mechanisms for retinal deposit.

5. AMD-related gene variation identified by single nucleotide polymorphism (SNP) association studies

Recently, studies have begun to provide evidence that genetic sequence variations may play important roles in the pre-symptomatic stages of AMD as well as in the specific pathogenesis of the different phenotypes of AMD. There are several types of genetic polymorphisms encountered in the human genome such as repeat polymorphisms, insertions and deletions. However, the majority of the DNA sequence variation in the human genome is in the form of SNPs. SNPs can be defined as persistent substitutions in at least one population of a single base with a frequency of more than 1%. SNP analysis has become an attractive tool in the exploration of the genetic component of complex diseases such as AMD because these polymorphisms are easily visualized and typed, highly abundant, and extremely stable. There are three principal investigative approaches taken when designing AMD-related SNP variation association studies: (1) to examine SNPs that have already been correlated with other age-related diseases that share similar pathological features with AMD; (2) to investigate SNPs in genes that have been implicated by other studies on monogenetic retinal and macular degenerative diseases as being AMD-related as described above; and (3) to focus on SNPs in genes that play roles in the proposed pathways involved in AMD development and pathogenesis.

5.1. AMD-related SNPs in genes associated with monogenic macular and retinal degenerative diseases

As mentioned above, the recent progress made in the study of hereditary monogenic macular and retinal dystrophies has offered some investigatory leads in the study of AMD genetics. The similarities that arise between the phenotypic expression in these early onset diseases and some of the later onset complex traits seen in AMD suggest a potential involvement of these candidate genes in AMD. Once candidate genes have been implicated as being AMD related, variation within these genes in the forms of SNPs can be studied. Table 2 summarizes the AMD SNP-related findings of association studies investigating the implications of other monogenic dystrophies studies in AMD genetic research.

Variation screening of *ELOVL4*, the gene identified as causal to STGD-like macular dystrophy, in AMD patients or additional families with dominant macular dystrophy phenotypes has not uncovered any other disease-associated variants (Ayyagari et al., 2001). *ELOVL4* encodes an enzyme presumably involved in the elongation of very long chain fatty acids. A total of 11 sequence variants were detected in the entire *ELOVL4* gene in AMD patients and age-matched controls. Three SNPs were detected in the non-coding region of the gene and eight amino acid changing variants were revealed in the coding region. The most common variant encountered was the *ELOVL4* M299V in exon 6 (Ayyagari et al., 2001). No statistically significant association was found between sequence variants in the *ELOVL4* gene and susceptibility to AMD with similar rare allelic frequencies in both AMD case and control populations (Ayyagari et al., 2001).

Data from several independent studies exclude the possibility of a major role for *VMD2*, the causative gene in juvenile-onset Best disease, in the genetic predisposition to AMD. Three independent groups have screened for variant *VMD2* genes in large numbers of AMD patients and matched control populations. No disease-associated alterations were detected in any of the 200 AMD patients involved in one study (Kramer et al., 2000). Furthermore, this study observed no association between the four common intragenic polymorphisms (221T → C, IVS4-24C → T, IVS-del (TCC)₃ and 1410A → G) and AMD (Kramer et al., 2000). Another study reported 40 possible *VMD2* mutations in patients with Best disease, 29 of which are novel, and the majority of which are only observed in patients with the Best disease phenotype. SSCP analysis only detected *VMD2* sequence variants in five out of the total 321 AMD patients (roughly 1.5%) included in this particular study (Lotery et al., 2000). Although no variants were detected in the 192 controls, this frequency difference is not significant enough to assume an association with confidence. A study of 259 patients with AMD found that allelic frequencies of the common polymorphisms associated with *VMD2* were almost identical in both the patient and control populations. However, two novel alterations, T216I and L567F, which were absent in the control population, were identified in three AMD patients with the sporadic dry form of AMD (Allikmets et al., 1999). Albeit intriguing, *VMD2* analysis in AMD patients has provided a small number of sequence variants with uncertain significance. Although, it is widely agreed that the *VMD2* gene variations studied thus far do not significantly contribute to AMD development, as the T216I and L567F alterations imply, bestrophin, the protein encoded by *VMD2*, variants may still predispose an individual to AMD in rare cases (Allikmets et al., 1999).

Metalloproteinases are secreted enzymes involved in the degradation and renewal of the extracellular matrix. Therefore, TIMPs have been associated with the regulation of extracellular matrix turnover. It has been observed that TIMP-3 content in Bruch's membrane is greatly elevated (roughly twofold) in AMD eyes as compared to matched control eyes ($p < 0.001$) (Kamei and Hollyfield, 1999). Overactive and accumulated TIMP-3 is believed to contribute to the thickening of Bruch's membrane and the associated atrophy of the RPE and photoreceptors seen grossly in AMD patients by inhibiting normal membrane turnover.

No sequence variants in the *TIMP3* coding region have been detected in AMD patients. Besides one sequence variation found in one patient with AMD, no other disease-causing mutations have been detected in this gene (Felbor et al., 1997). Familial linkage studies have also failed to implicate *TIMP3* in AMD (De La Paz et al., 1997). Although no association has been established between AMD and the *TIMP3* gene variations, it may be possible that a variation within the genes that regulate *TIMP3* translation causes the observed protein-level elevation.

As previously mentioned, a single base change within the *EFEMP1* gene has been correlated with *Malattia leventinese* and Doyme honeycomb retinal dystrophy. Variations within this gene result in a misfolded protein that is poorly secreted. Retarded secretion leads to aberrant accumulation and retention within cells. Defective protein aggregates are a common feature among many degenerative diseases. *EFEMP1* has been shown to play an important role in drusen formation and cellular degradation. However, the Arg345Trp variant of the *EFEMP1* gene does not appear to be associated with cases of early onset drusen that fall outside of these two diseases, nor does this variation appear to play a major role in AMD (Guymer et al., 2002). Out of the 494 AMD patients included in one study, neither mutations nor any abnormalities were found after screening the entire *EFEMP1* coding region (Stone et al., 1999). Involvement of the Arg345Trp variant has been found to be absent in sporadic cases of AMD (Stone et al., 1999). *EFEMP1* also does not appear to be correlated with familial AMD (Guymer et al., 2002). SSCP analysis has determined that the variant is not evident in control populations, suggesting that this is a disease-causing allele mutation rather than a polymorphism (Guymer et al., 2002). Further studies are required in order to determine the role *EFEMP1* plays in the etiology and pathology of AMD.

Human G protein coupled receptor-75 (GPR75) is a two-exon gene that encodes a G-protein coupled receptor whose neuronal expression is restricted primarily to the retina and brain. GPR75 was originally studied during the hunt for the causal gene of Doyme honeycomb retinal dystrophy and *Malattia leventinese* prior to the establishment of *EFEMP1*'s role in these disorders (Sauer et al., 2001). Genetic screening, however, revealed no association between sequence variations within this gene and the development and pathogenesis of Doyme honeycomb retinal dystrophy or *Malattia leventinese*. GPR75 is a member of the superfamily of transmembrane proteins that mediate signal transduction. Rhodopsin is also a member of this family. The potential functional properties of GPR75 and its expression in the retinal tissue have made this gene a candidate in AMD association studies. An analysis of 535 unrelated AMD patients and 252 matched controls identified a total of nine sequence alterations within this gene, three of which are believed to be polymorphic (Sauer et al., 2001). However, no statistical significance between these sequence variants and AMD risk was observed.

Schultz et al. (2003b) screened the single nucleotide polymorphism (SNP) (*hemicentin - 1* - Gln5345Arg) in sporadic AMD cases and found a very low frequency of 5345Arg (1/188). However, they also found a similarly low frequency in random control subjects (2/174). Two 5345Arg carriers (aged 57 and 64 years) were found in the control population. One 5345Arg carrier (age 74 years at the time of diagnosis) was identified in the AMD cases (Schultz et al., 2003b). There are four *hemicentin - 1* SNPs that are reported (Iyengar et al., 2004). Among the four, rs1475113, rs743137 and rs680638 account for the majority of the linkage (Section 3). The findings of this report further suggest that variants within or in very close proximity to this gene could be a factor in AMD pathogenesis. To what degree and by which mechanisms *hemicentin - 1* plays a role in AMD pathogenesis are questions that remain to be answered.

A total of 43 sequence variants have been described in the *human peripherin / RDS* gene. Thirty-nine of these variants have been associated with retinal and macular phenotypes. The mutations associated with severe macular degeneration have been localized between the third and fourth transmembrane domains. Three of the amino acid polymorphisms of this gene,

Glu338Gln, Lys310Arg, and Gly33Asp have a genotype frequency of approximately 50% in the normal population (Keen and Inglehearn, 1996). These polymorphisms are located on the third exon of the *peripherin / RDS* gene. Silvestri (1997) screened 50 AMD patients and matched controls for variations within the *peripherin/RDS* gene. No disease-causing alteration was detected in either the case or control populations. Although associations have been made between *peripherin / RDS* mutations and polymorphisms and other retinal dystrophies, no similar associations have been found between these polymorphisms and AMD (Silvestri, 1997).

Although several genes have been identified as causative in other human macular disease phenotypes, none, with one possible exception, have been found in association with AMD. This exception is the causal gene of autosomal recessive juvenile-onset STGD1, *ABCA4*. The role of *ABCA4* in relation to AMD has been the subject of much debate and the focus of extensive study. These studies have generated a long series of conflicting reports. Heterozygous mutations in *ABCA4* have been associated with AMD in several studies (Allikmets et al., 1997a; Allikmets, 2000; Mata et al., 2001; Shroyer et al., 2001; Zhang et al., 1999); however, the significance of this association has been challenged.

The *ABCA4* controversy began in 1997 when Allik-mets et al. (1997a) reported 13 different AMD-associated gene variations in one allele of *ABCA4*. The AMD-associated alterations were scattered throughout the coding sequence of the *ABCA4* gene, although more were located towards the 3' end. In their first study, 167 unrelated patients with AMD were screened, 26 (a significant 16%) of whom were reported to carry a suspected disease-associated variation (Allikmets et al., 1997a). The most common *ABCA4* variants identified by Allikmets et al. were D2177N and G1916E. The D2177N variant was reported as being significantly associated with AMD risk ($p = 0.023$). Allikmets et al. (1997a,b) convincingly proposed that mutations in the *ABCA4* are associated with an increased risk of AMD. Allikmets later retested this hypothesis, that *ABCA4* is a dominant susceptibility locus for AMD, by screening 1218 unrelated AMD patients and 1258 controls for the two most frequent sequence variants, G1961E and D2177N. This study reported a statistical significance ($p < 0.0001$) between these two variants and AMD risk (Allikmets, 2000). Furthermore, they reported that the risk of AMD is elevated approximately threefold in D2177N carriers and approximately fivefold in G1961E carriers (Allikmets, 2000). Further support was granted by a cohort study carried out by Shroyer et al. (2001) in which a complete sequence analysis of the *ABCA4* gene and tests of cosegregation in 22 families that manifest both STGD1 and AMD were carried out. Data from this study showed that STGD-causing mutations in *ABCA4* are associated with AMD (with a higher rate of AMD parents and grandparents of patients with STGD1 harboring *ABCA4* variants, $p=0.038$) and that AMD-associated mutant *ABCA4* proteins have substantive defects in both expression and ATP binding (Shroyer et al., 2001). Additionally, Mata et al. (2001) developed a mouse model that shows that the absence of one *ABCA4* allele can lead to retinal degeneration. In this mouse model, mice heterozygous for *ABCA4* accumulate A2E at a slower age-related rate and show severely delayed dark adaptation, a phenomenon which provides evidence of photoreceptor degradation and lipofuscin accumulation in the RPE (Mata et al., 2001). A2E is a major component of lipofuscin and an inhibitor of lysosomal proteolysis in RPE cells. A2E formation and accumulation occur as the result of *ABCA4* dysfunction or variation. A2E acts as a cationic detergent and dissolves cellular membranes when over-expressed in high concentrations (Mata et al., 2001). This accumulation may be deleterious to the RPE and to photoreceptors. A2E has also been shown to self-generate singlet oxygen upon irradiation which reacts with A2E to produce epoxides that damage DNA (Beatty et al., 2000).

Despite these findings, there have been several studies that did not find any statistical evidence for an association between either of the G1961E and D2177N alleles and AMD. Guymer et al.

(2001) found no significant difference ($p > 0.1$) between 544 AMD patients and 689 controls. Furthermore, no evidence of cosegregation of these alleles and the AMD phenotype was observed in the examined five multiplex families with AMD. They did, however, find that the two *ABCA4* variant alleles were slightly more frequent in patients with the exudative form (or wet-type) of AMD versus those without this complication (2.7% compared to 2.5%, respectively) (Guymer et al., 2001). De La Paz et al. (1999) observed rates of *ABCA4* variants significantly lower (1.2%) than the rates Allikmets et al. reported (16%, $p = 0.00001$; see above). Furthermore, the D2177N variant was only reported in two AMD sporadic individuals and this allele was not found in any of the familial AMD cases in this study. Interestingly, De La Paz et al. (1999) did report that a newly identified polymorphism, T190A, was found among all affected members of one of their larger AMD families although it failed to definitively segregate with the disease phenotype in another family. Similarly, several other independent reports found no evidence for significant association or cosegregation with the disease in family-based studies (Rivera et al., 2000; Schmidt et al., 2003; Souied et al., 2000; Stone et al., 1998; Webster et al., 2001). Thus, the true nature of *ABCA4* variation as a potential risk factor for AMD needs to be further evaluated.

5.2. SNPs associated with the oxidative stress AMD pathway

Oxygen consumption in the retina is much higher than in other human tissues (Beatty et al., 2000). The free radical theory of aging purports that age-related disorders arise as a result of oxidative stress, or the collective cellular damage caused by reactive oxygen species (ROS) (Beatty et al., 2000; Frank, 1998; Frank et al., 1999). The retina is particularly vulnerable to oxidative damage because of its high consumption of oxygen, the high proportion of polyunsaturated fatty acids in the outer segments of the RPE cells, and its high exposure to irradiation by visible light (Beatty et al., 2000). Thus, oxidative stress could potentially be a major pathway in AMD pathogenesis. Oxidized LDL levels are significantly higher in the plasma of AMD patients as compared to control subjects (Ikeda et al., 2001). In the presence of specific risk factors, genetic and/or environmental, an excess of oxidative damage may lead to pathological changes and disease. It has been hypothesized that the pathogenesis of AMD may involve the failure of the normal "oxidative protective" enzymes and mechanisms within the RPE to defend the macula against such stresses. Genetic variations within the antioxidants and mechanisms that provide protection against the effects of oxygen toxicity have been the focus of many AMD genetic association studies (For a summary, refer Table 3).

Kimura et al. (2000) screened for polymorphic xenobiotic-metabolizing and antioxidant enzymes in Japanese populations afflicted with exudative (or wet-type) AMD. No association was established between AMD and the two polymorphisms, one located in the 3' -flanking region and one in exon 4, of the *cytochrome P-450 1A1* gene. Also, no association was established between AMD and the *glutathione S-transferase theta 1*, *glutathione S-transferase mu 1*, and *microsomal epoxide hydrolase exon-4* polymorphisms. Allele frequency distribution of all five of these polymorphisms in AMD cases was similar to that found in the Japanese and Caucasian control populations (Kimura et al., 2000).

Kimura et al. (2000) did, however, report a statistically significant association between a polymorphism in the antioxidant enzyme manganese *superoxide dismutase* gene (*MnSOD*) and the exudative form of AMD. *MnSOD* is an isoform of *superoxide dismutase 2* (*SOD2*). This gene encodes an intramitochondrial free radical scavenging enzyme preferentially expressed in the retina and believed to play an important role in tissue and cell protection against oxidative stress. Variation in this gene has been implicated in a wide range of degenerative processes. The *MnSOD* polymorphism, which involves the substitution of a C from T at the -9 position in the mitochondrial targeting sequence, results in an increased and more efficient transport of the MnSOD protein into the mitochondria. The mitochondrion, a

major producer of ROS, is itself subject to direct attack by these damaging radicals and is the main target of oxidative injury. This *MnSOD* polymorphism causes a higher basal level of activity that results in excess hydrogen peroxide. The hydrogen peroxide then reacts with ferrous iron to form a surplus of cytotoxic hydroxyl radicals. These radicals can attack mitochondria and may promote the development of wet AMD. The same study also reported a weak association between *microsomal epoxide hydrolase exon-3 (MEHE-3)* and AMD (Kimura et al., 2000). The MEHE-3 enzyme catalyzes the hydrolysis of the epoxides derived from the oxidative metabolism of xenobiotic chemicals and pollutants. Both the *MnSOD* and *MEHE-3* genes are expressed in the retina and the RPE cells (Behndig et al., 1998).

SNP association studies have also been carried out investigating the *paraoxonase* polymorphism. Immunohistochemical studies suggest that oxidative modified lipids and proteins play important roles in the pathogenesis of AMD. Paraoxonase is a polymorphic protein known to affect lipid metabolism as well as play a protective role against oxidative stress (Garin et al., 1997; Mackness et al., 1991; Ikeda et al., 2001). Specifically, paraoxonase is associated with high-density lipoprotein and has been shown to prevent LDL oxidation. Paraoxonase is a calcium-dependent glycoprotein. Two *paraoxonase* polymorphic sites, Glu192Arg and Leu54Met, have been studied in association with AMD. Both of these polymorphisms lead to different enzymatic activities, with the variant genotypes being associated with higher serum concentrations of the enzyme and, therefore, higher paraoxonase activity as well (Garin et al., 1997; Ikeda et al., 2001). Both the Glu192Arg and Leu54Met polymorphisms have been reported as being significantly associated with the risk of developing wet-type AMD in Japanese populations ($p = 0.0445$; and 0.0323 , respectively) (Ikeda et al., 2001).

Metallothioneins (MTs) are also involved in the retinal antioxidant defense system. MTs, in their capacity as acute phase stress proteins, are believed to act as scavengers of active oxygen species and various radicals and protect cells from peroxidation (Sato et al., 2000). This protein also is believed to participate in the detoxification, metabolism, and homeostasis of some heavy metals, namely zinc, copper, and cadmium by binding and releasing them. MTs have also been implicated in the regulation of the cell proliferation process. It has been demonstrated that there is an age-related decline noted in the human macular RPE content of MTs (Beatty et al., 2000). At least six polymorphisms in the coding region of the *MT II* gene and three in the *MT IA* gene were found in the Japanese patients (Sato et al., 2000). However, no amino acid substitution was observed in these genes in Japanese patients nor was statistical significance established (Sato et al., 2000).

Efficient DNA repair mechanisms, particularly in the mitochondria, are necessary to maintain cellular integrity after oxidative damage has occurred (Liang and Godley, 2003). The disruption of DNA repair genes often leads to degenerative and earlier-onset age-related diseases such as the Werner and Bloom Syndromes. The *human Ogg1 (hOgg1)* gene encodes a DNA glycosylase that is involved in the base excision repair of 8-hydroxy-2'-deoxyguanine (8-OH-dG) from oxidatively damaged DNA. The Ser326Cys of *hOgg1* SNP has been reported in association with certain kinds of cancer. The *CSB* gene, also called *ERCC6*, is involved in the preferential repair in eukaryotes and collaborates with *hOgg1* to carry out DNA repair (Tuo et al., 2001, 2002a,b, 2003). The *CSB* gene also plays a role in the mitochondrial repair of oxidative DNA damage. In our preliminary studies, no association has been found between either the Ser326Cys of *hOgg1* or the *CSB*-promoter-6530C/G sequence variations and AMD (Bojanowski, unpublished data).

5.3. SNPs that confer protection against AMD

There have been reports on SNPs in genes believed to confer protection against AMD development (summarized in Table 4). The Alu polymorphism in the *angiotensin - converting*

enzyme (ACE) gene, localized to the retina, has previously been associated with the ocular disease proliferative diabetic retinopathy. Proliferative diabetic retinopathy is characterized by neovascular complications. For this reason, the *ACE* polymorphism was hypothesized to be associated with the wet, or exudative, form of AMD (Hamdi et al., 2002). *ACE* is an enzyme known to catalyze the conversion of angiotensin I to angiotensin II. This enzyme is involved in electrolyte balance and blood pressure regulation and is implicated in cell proliferation and death in development and disease processes. This polymorphism, caused by an Alu insert, is believed to be responsible for the variable circulating level of *ACE*. No association was found between the *ACE* allelic and genotypic frequencies and the AMD case populations when compared to the control subjects. There was also, interestingly enough, no significant difference between the control and the neovascular/wet form of AMD case populations (Hamdi et al., 2002). However, upon further stratification, Hamdi et al. (2002) did find that the *ACE* Alu+/+ genotype was 4.5 times more frequent in the control population than in the dry/atrophic form of AMD. Although, the exact mechanisms by which this *ACE* polymorphism might be linked to the pathology of AMD is not yet understood, these findings suggest a possible protective role of the polymorphism against the dry/atrophic form of AMD (Hamdi et al., 2002).

ApoE is an important regulator of cholesterol and lipid clearance and transport. This gene plays a pivotal role in the processes following central nervous system (CNS) injury, as it is responsible in part for neural plasticity and neuronal cell membrane maintenance and repair (Boyles et al., 1989; Poirier et al., 1993). Synthesized by astrocytes, the major glial cell of the CNS, ApoE is a major component of plasma and cerebrospinal fluid. ApoE or ApoE receptor dysfunction results in the accumulation of lipoproteins under certain conditions (Laws et al., 2003). The *ApoE* gene is known to be polymorphic (containing as many as 21 SNPs) with the occurrence of three common alleles; ApoE ϵ 2, ApoE ϵ 3, and ApoE ϵ 4 (Laws et al., 2003). The most common allele, the ϵ 3 allele, is considered to be the ancestral allele while the ϵ 2 and ϵ 4 alleles are considered as variants. These alleles encode three major isoforms ϵ 2 (Cys112, Cys158), ϵ 3 (Cys112, Arg158), ϵ 4 (Arg112, Arg158) which vary greatly in terms of protein structure and function (Laws et al., 2003; Schultz et al., 2003a).

The *ApoE* gene polymorphisms have been associated with genetic risk factors of various neurodegenerative diseases, most notably Alzheimer Disease (AD) (Laws et al., 2003). The epidemiological association of AMD to AD and similarities of some pathological features sparked the interest of the possible involvement of the *ApoE* gene in AMD (Klaver et al., 1998a, 1999). It has been hypothesized that the neuronal degeneration occurring in AMD and AD may, to some extent, have a common pathogenesis based on the recent finding of amyloid beta ($A\beta$), the major extracellular deposit in AD plaques, in drusen, the early hallmark of AMD (Dentchev et al., 2003). $A\beta$ is hypothesized to increase the oxidative stress and inflammation expressed in AMD as it does in AD plaques (Dentchev et al., 2003). ApoE has also been shown to be a ubiquitous component of soft drusen, which arises from protein and lipid deposits within Bruch's membrane and is expressed in high concentrations in eyes affected by retinal damage (Dentchev et al., 2003). ApoE, therefore, is an intuitive candidate gene for AMD genetic studies both for its implicated role in AD and for its role in lipid metabolism.

Although there have been some contradictory reports (Schultz et al., 2003a), it is widely accepted that *ApoE* is a susceptibility gene for AMD. The ApoE ϵ 4 isoform has been previously correlated with elevated cholesterol concentrations, stroke, and increased risk of coronary heart disease (Souied et al., 1998; Schultz et al., 2003a,b). However, ϵ 4 has been reported in association with a decreased risk of exudative AMD with soft drusen, with the ϵ 4 allele reported as being significantly less prevalent among wet AMD cases as compared with control populations (Klaver et al., 1998b; Schultz et al., 2003a; Souied et al., 1998). Klaver et al. (1998b)

calculated that individuals that carry the ϵ 4 isoform were greater than 2 times less likely to develop AMD than were subjects with the ϵ 2 ϵ 3 genotype.

There have been two other studies that have further narrowed the proposed protective effect of the ϵ 4 allele. Schultz et al. (2003a) observed a trend toward a lower frequency of the ϵ 4 allele in only unrelated individuals with a family history of AMD. Later, Schmidt et al. (2000) found no evidence of ϵ 4 association with sporadic cases of AMD and further suggested that the protective effect of the ϵ 4 allele is restricted to familial AMD cases only. While the ϵ 4 polymorphism is associated with a protective effect against AMD, the ϵ 2 allele has been associated with a slightly increased risk of late AMD. Studies have reported a weak causative role for the ϵ 2 allele in AMD with the frequency of the ϵ 2 allele higher, although not significantly, in AMD subjects (Klaver et al., 1998b; Simonelli et al., 2001). There has been no evidence for either a risk-increasing or risk-decreasing effect attributed to the ϵ 3 allele in either familial or sporadic cases of AMD.

5.4. CX3CR1—implications for the possible immunological mechanisms behind AMD development and pathogenesis

Recently, investigators have looked into the immunological mechanisms hypothesized to be involved with the development and pathogenesis of AMD. Macrophage dysfunction in clearing retinal deposits through the influence of chemoattraction has been a topic of particular interest and discussion (Ambati et al., 2003b; Espinosa-Heidmann et al., 2002). CX3CR1, a CX3C chemokine receptor of fractalkine, is known to be polymorphic with two non-synonymous SNPs in the open reading frame, V249I and T280M. Both of these variations ultimately result in reduced chemoattraction by lowering the binding affinity of the receptor to fractalkine and diminishing the number of available binding sites (McDermott et al., 2003).

In a preliminarily population-based study, we have found an association between the sequence variation of *CX3CR1* (V249I and T280M) and AMD risk. CX3CR1-249I and 280M show an increased prevalence among AMD cases as compared to controls in a multiple control study (19.5% 280M carriers in AMD over 11.4% 280M carriers in control, OR=1.81; $p < 0.05$) (Tuo et al., 2004). Furthermore, a dose-response between 249I/280M and AMD was demonstrated with 3.28 as the highest odds ratio. We compared the expression of *CX3CR1* mRNA in AMD macular and peripheral retinal cells from archived paraffin embedded slides. The transcripts of *CX3CR1* were undetectable in macula lesions but detectable in the normal peripheral retina cells (Tuo et al., 2004). While associations between 249I/280M and AMD exist, it seems unlikely that these variants affect the level of transcription directly. If the level of transcription is a true susceptibility factor, then the determinants may lie in the regulatory sites of *CX3CR1*, possibly in linkage disequilibrium with V249I and T280M. These variations might then serve as an intermediate trait. With such promising initial findings, investigating genes involved in the immunological mechanisms implicated in AMD development and pathogenesis has become an interesting potential avenue of research for future association studies.

These SNP association studies highlight some of the critical challenges that investigators face attempting to uncover the genetic component of not only AMD, but the genetic component of any complex disease. Complex diseases are marked by genetic heterogeneity, low penetrance, a continuous phenotypic distribution, and a high susceptibility to non-genetic factors. Failure to replicate results by independent groups is a major obstacle for advancement. Despite the limitations and difficulties historically associated with SNP analysis, there has been a renewed emphasis on association studies as they yield greater statistical power than linkage in the identification of disease gene loci of small effect. We must continue to rely on association studies to identify candidate genes and to test out various hypotheses.

6. Perspectives

Uncovering the genetic risk factors for AMD is imperative for future progress in the battle against this disorder. Identification of AMD's genetic causes will allow investigators and clinicians to develop better definitions and characterizations of the disease phenotypes. In addition, determination of genetic risk factors may contribute to a better understanding of the pathogenesis of this disease. These advances will greatly impact clinical treatment as well as direct the application of molecular manipulation and preventive interference. Progress has already been made in the development of potential treatments based on several of the factors that have been reviewed in this paper. For example, based on the correlation of the *peripherin/RDS* with retinitis pigmentosa and AMD, subretinal injections of recombinant adeno-associated virus encoding a *peripherin/RDS* transgene have been investigated with promising findings in mice (Ali et al., 2000). These injections result in the generation of outer-segment structures and the formation of new stacks of discs containing both *peripherin/RDS* and rhodopsin thus preserving electrophysiological functions in retinal degeneration slow (*peripherin/RDS* null) mice. Another potential treatment involves the oral administration of long chain fatty acids to AMD patients based on the studies of *ELOVL4* in AMD (Ayyagari R., NEI Seminar 2003).

During the preparation for this review, it became evident that there is no consensus in the ophthalmologic and genetic communities regarding the identification of definitive AMD-related loci, genes, and sequence variants. The AMD loci obtained from whole genome scans cover only a fraction of the genes identified in monogenic retinal diseases, animal models, and gene variation studies (see Tables 1–4). Sequence variants in the genes identified from monogenic retinal and macular disorders have not proven to be associated with AMD, with the possible exception of *ABCA4*. Progress in the study of AMD genetics has been delayed and greatly confounded by the intrinsic complex nature of the disease and the considerable impact of environmental factors on disease development and progression. Examples of the difficulties involved in conducting these genetic studies include; varying phenotypes, continuous distribution within a phenotype, uncertain modes of inheritance, low lod scores in linkage, similar linkage evidence but in non-identical loci from different studies, and continuous distribution of associated traits (SNP) in AMD cases as well as in controls. In addition, the disease is also strongly influenced by, and interacts with, many non-genetic factors. Clearly, the solution to the AMD genetic problem will be much harder to solve than we imagined.

Even though results have been controversial and inconsistent so far, it is generally believed that association studies are still applicable to complex diseases such as AMD. While only a limited number of related genes and SNPs within genes have been tested for association with AMD, the current lack of evidence for association does not completely exclude the involvement of these genes in AMD development. Bias is another problem of AMD association studies. However, bias can be limited by careful study design. Since AMD is age-dependent and of a high incidence, multiple control groups with different average ages can be used to elucidate a possible dose-response of the tested genetic risk. This design is compensatory in studies with small sample sizes. Well-defined phenotyping, disease scoring, and continuous studies in a cohort will also be invaluable to the quantitative analyses of genetic donation and stratification. In addition, efficient information can be obtained from a well-matched case-control study with minimal ethnic differences. Finally, family-based designs that involve typing both affected and consanguineous individuals could also remove the possible confounding factor of ethnicity (Brennan, 2002).

In AMD, as in other complex diseases, the role of genetics is thought to be the result of the combined effects exerted by multiple gene variants and their interaction with environmental

factors. Therefore, the interaction between the environment and genes cannot be ignored during our search for AMD gene variants. Environmental components could trigger the AMD phenotype in genetically predisposed individuals. A family-based design with spouse involvement could be an effective way to limit confounding environmental factors. Quantitative interpretations of the confounders could greatly help in the search to determine the extent that these environmental factors contribute to the development of AMD.

Interestingly, many products of the genes discussed above were detected either by immunohistochemistry or proteomics in human AMD drusen specimens or animal models. These include TIMP3, ApoE, collagen, etc. (Ambati et al., 2003b; Crabb et al., 2002; Mullins et al., 2000). The elucidation of the molecular mechanisms of the accumulation of the gene products in the retina could further confirm or obviate the roles of these genes in AMD.

One of our aims is to genetically screen individuals at an early age and assess their genetic predisposition of developing AMD in order to provide effective interference at an optimal time. If genetic predisposition is discovered at an early age, behavioral styles can be modified to curtail or even completely avoid the devastating effects of macular degeneration later in life. Current evidence has shown that it seems highly unlikely that a single gene variant is solely responsible for the development of AMD, at least not in the common forms of the disease. Perhaps focusing on the dynamic interaction between multiple genes, gene-environmental components and their comprehensive effects will provide new insights in the search for the genetic risks, pathogenesis and treatment of AMD.

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Table 1AMD-related chromosomal loci with lod ≥ 2

AMD locus	Marker	Maximum lod	References
1q25-31	D1S240/D1S412	3.20	Klein et al. (1998),Majewski et al. (2003),Seddon et al. (2003),Weeks et al. (2001),Iyengar et al. (2004)
2q31/2q32	D2S1391/D2S1384	2.32/2.03 ^{aa}	Seddon et al. (2003)
3p13	D12S1300/D12S1763	2.19 ^b	Majewski et al. (2003),Schick et al. (2003)
6q14	D6S1056/D6S249	3.59/3.17	Kniazeva et al. (2000)
9q33	D9S934/D9S934	2.06	Majewski et al. (2003)
10q26	D10S1230	3.06	Majewski et al. (2003),Kenealy et al. (2004),Iyengar et al. (2004)
17q25	D17S928	3.16	Weeks et al. (2001)
22q12	D22S1045	2.0	Seddon et al. (2003)

^aNon-parametric Z score calculated by Genehunter software and is considered as extremely conservative (Seddon et al., 2003).

^bHighly associated with 10q26.

Table 2
Causative genes of other retinal and macular dystrophies and their relationship with AMD

Gene	Locus	Implicated diseases	AMD-association findings	Studies
<i>VMD2</i>	11q13	Best disease	No significant association	White et al. (2000), Allikmets et al. (1999), Kramer et al. (2000), Lotery et al. (2000)
<i>EFEMP1</i>	2p16	<i>Malattia leventinese</i> and Doyne Honeycomb Retinal Dystrophy	No changes found	Li et al., (2001), Zhang et al. (2001)
<i>GPR75</i>	2p16		No statistical evidence for an association between the variants and AMD	Sauer et al. (2001)
<i>TIMP3</i>	22q12.1–q13.2	Sorsby Fundus Dystrophy	No evidence of sequence variation in AMD patients. Confirmed by linkage studies	Felbor et al. (1997)
<i>Peripherin-RDS</i>	6p21	Pattern Dystrophies, progressive macular degeneration, dominant retinitis pigmentosa (RP), digenic RP	No association found. Polymorphisms are equally common in both AMD case and matched control populations	Silvestri (1997), Keen and Inglehearn (1996)
<i>ABCA4</i> (<i>ABCR</i>)	1p21–p22	Stargardt-like disease	No significant difference found among different allele frequencies and ABCR sequence variants in AMD patients and controls	Souied et al., (2000), Stone et al. (1998), Guymer et al. (2001), Klaver et al. (1998a), Michaelides et al. (2003), Rivera et al. (2000), Schmidt et al., 2003, Webster et al. (2001)
<i>ELOVL4</i>	6q14	Dominant Stargardt disease	Significant association established between ABCR variants (in particular, D1277N and G1961E) and AMD risk in matched control meta-analyses and cohort studies No statistically significant difference in <i>ELOVL4</i> variant allele frequencies between AMD patients and age-matched controls	Allikmets et al., (1997a), Allikmets (2000), Mata et al. (2001), Shroyer et al. (2001), Zhang et al. (1999) Ayyagari et al. (2001)

Table 3**Oxidative stress associated SNPs and AMD**

Gene and SNP	Locus	AMD-association findings	Studies
Cytochrome P-450 1A1	15q22-q24	No association found in Japanese population	Kimura et al. (2000)
Microsomal epoxide hydrolase exon-4 (MEHE-4)	1q42.1	No association found in Japanese population	Kimura et al. (2000)
Glutathione S-transferase theta1	22q11.23	No association found in Japanese population	Kimura et al. (2000)
Glutathione S-transferase mu 1	1p13.3	No association found in Japanese population	Kimura et al. (2000)
SOD2, manganese superoxide dismutase (MnSOD) isoform	6q25	Significant association of MnSOD with AMD ($p = 0.0005$ after Bonferroni correction). Increased frequency of alanine allele and alanine/alanine genotype	Kimura et al. (2000)
Microsomal epoxide hydrolase exon-3 (MEHE-3)	1q42.1	Weak association (odds ratio 2.2, $p = 0.02$ after Bonferroni correction)	Kimura et al. (2000)
Paraoxonase	7q21.3	Association found between both GlnArg192 and Leu54Met polymorphisms and AMD in Japanese populations (192 $p = 0.0445$; 54 $p = 0.0323$)	Ikeda et al. (2001)
Metallothionein	16q13	No association found	Sato et al. (2000)

Table 4

Protective SNPs against AMD

Gene	Locus	AMD-association findings	Studies
Apolipoprotein E (ApoE) E2, E3, E4	19q13.2	The frequency of the E4 allele carriers was significantly higher in the general population than in AMD patients ($p = 0.002$). The frequency of the E2 allele was higher in the patients ($p = 0.069$)	Simonelli et al. (2001a)
		No significant association found. Trend for a decreased risk of AMD associated with APOE4 observed in unrelated patients with a family history of AMD	Schultz et al. (2003a)
		Significant association of E4 allele with decreased risk of AMD (odds ratio 0.43). E2 allele associated with slightly increased risk for AMD (odds ratio 1.5)	Klaver et al. (1998b)
Angiotensin-converting enzyme (ACE), Alu+/+ insert	17q23	Lower frequency of E4 allele carriers observed in exudative AMD ($p < 0.0009$) less frequent in AMD group with control subjects ($p < 0.006$)	Souied et al. (1998)
		No significant difference observed between the controls and AMD populations of allelic and genotypic frequencies. However, the Alu+/+ genotype occurred 4.5 times more frequently in the control population than the dry/atrophic AMD population	Hamdi et al. (2002)