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Convergence of linkage, gene expression and association data demonstrates the influence of the RAR-related orphan receptor alpha (*RORA*) gene on neovascular AMD: A systems biology based approach

Alexandra C. Silveira [a], Margaux A. Morrison [a], Fei Ji [b], Haiyan Xu [b], James B. Reinecke [c], Scott M. Adams [a], Trevor M. Arneberg [a], Maria Janssian [a], Joo-Eun Lee [a], Yang Yuan [c], Debra A. Schaumberg [d], Maria G. Kotoula [e], Evangeline E. Tsironi [e], Aristoteles N. Tsiloulis [e], Dimitrios Z. Chatzoulis [e], Joan W. Miller [f], Ivana K. Kim [f], Gregory S. Hageman [g], Lindsay A. Farrer [h], Neena B. Haider [c], Margaret M. DeAngelis [a,*]

[a] *Ocular Molecular Genetics Institute and Department of Ophthalmology, Harvard Medical School, Massachusetts Eye and Ear Infirmary, Boston, MA, United States*
[b] *The Laboratory of Statistical Genetics, Rockefeller University, New York, NY, United States*
[c] *Department of Genetics, Cell Biology, and Anatomy, University of Nebraska Medical Center, Omaha, NE, United States*
[d] *Division of Preventive Medicine, Brigham and Women's Hospital, Boston, MA, United States*
[e] *Department of Ophthalmology, University of Thessaly School of Medicine, Larissa, Greece*
[f] *Retina Service and Department of Ophthalmology, Harvard Medical School, Massachusetts Eye and Ear Infirmary, Boston, MA, United States*
[g] *Department of Ophthalmology and Visual Sciences, Cell Biology and Functional Genomics Laboratory, The University of Iowa, Iowa City, IA, United States*
[h] *Departments of Medicine (Genetics), Neurology, Genetics and Genomics, Epidemiology, and Biostatistics, Boston University Schools of Medicine and Public Health, Boston, MA, United States*

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ABSTRACT

To identify novel genes and pathways associated with AMD, we performed microarray gene expression and linkage analysis which implicated the candidate gene, retinoic acid receptor-related orphan receptor alpha (*RORA*, 15q). Subsequent genotyping of 159 *RORA* single nucleotide polymorphisms (SNPs) in a family-based cohort, followed by replication in an unrelated case-control cohort, demonstrated that SNPs and haplotypes located in intron 1 were significantly associated with neovascular AMD risk in both cohorts. This is the first report demonstrating a possible role for *RORA*, a receptor for cholesterol, in the pathophysiology of AMD. Moreover, we found a significant interaction between *RORA* and the *ARMS2/HTRA1* locus suggesting a novel pathway underlying AMD pathophysiology.

1. Introduction

The etiology of age-related macular degeneration (AMD) has yet to be fully elucidated; nevertheless, it is clear that the development and progression of this complex, multifactorial disease may be influenced by several different pathways, including cholesterol and lipid metabolism (for reviews please see Ding, Patel, & Chan, 2009; Javitt & Javitt, 2009). Epidemiologic findings have indicated a role for lipid/cholesterol metabolism in the pathogenesis of AMD (Baker et al., 2009; Klein, Klein, & Jensen, 1997; Tan, Wang, Flood, & Mitchell, 2009). This is further supported by evidence that comes from studies showing that the use of cholesterol lowering drugs (statins) has a protective effect against the development of neovascular AMD and all types of AMD (McGwin, Xie, & Owsley, 2005; Wilson, Schwartz, Bhatt, McCulloch, & Duncan, 2004); however, others have found no significant association between use of these drugs and any AMD subtypes (Klein, Klein, Tomany, Danforth, & Cruickshanks, 2003; van Leeuwen, Vingerling, Hofman, de Jong, & Stricker, 2003). Additionally, both *in vivo* and *in vitro* assays have implicated a role for cholesterol/lipid metabolism in the development of AMD (Mullins, Russell, Anderson, & Hageman, 2000; Sallo et al., 2009; Yamada et al., 2008; Yu, Lorenz, Haritoglou, Kampik, & Welge-Lussen, 2009; for review please see Ding et al., 2009).

Genetic studies have similarly implicated several lipid/cholesterol metabolism and transport genes in the pathophysiology of early and/or advanced stages of AMD. For example, the genes toll-like receptors -3 and -4 (Yang et al., 2008; Zareparsi et al., 2005), apolipoprotein E (Anderson et al., 2001; Baird, Guida, Chu, Vu, & Guymer, 2004; Klaver et al., 1998; Schmidt et al., 2002;

* Corresponding author. Address: Department of Ophthalmology, Harvard Medical School, Massachusetts Eye and Ear, 243 Charles Street, Boston, MA 02114, United States. Fax: +1 617 573 4352.
E-mail address: margaret_deangelis@hms.harvard.edu (M.M. DeAngelis).

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Souied et al., 1998; Zareparsi et al., 2004), ATP-binding cassette transporter (Allikmets, 2000; Allikmets et al., 1997) and the elongation of very long chain fatty acids-like 4 (Conley et al., 2005) have all been associated with risk of all AMD subtypes. However, these findings have not been replicated consistently (Allikmets et al., 2009; Ayyagari et al., 2001; Cho, Chew, Mitchell, & Tuo, 2009; De La Paz et al., 1999; DeAngelis et al., 2007; Despriet et al., 2008; Edwards, Swaroop, & Seddon, 2009; Edwards et al., 2008; Guymer et al., 2001; Haddad, Chen, Santangelo, & Seddon, 2006; Lewin, 2009; Liew, Mitchell, & Wong, 2009; Schultz et al., 2003; Souied et al., 2000; Stone et al., 1998).

The variants and haplotypes most consistently associated with AMD are within the gene complement factor H (*CFH*) (1q32) and the locus containing the genes age-related maculopathy susceptibility 2 and HtrA serine peptidase 1 (*ARMS2* and *HTRA1*) (10q26) (DeAngelis et al., 2008; Dewan et al., 2006; Edwards et al., 2005; Hageman et al., 2005; Haines et al., 2005; Jakobsdottir et al., 2005; Kanda et al., 2007; Klein et al., 2005; Li et al., 2006; Rivera et al., 2005; Yang et al., 2006). These genes have been shown to have large influences on AMD risk in populations of various ethnicities, with variants on 10q26 being the most strongly associated with the neovascular AMD subtype (Fisher et al., 2005; Shuler et al., 2007; Zhang et al., 2008). Despite their large influence on AMD risk, the combination of these genes alone is insufficient to correctly predict the development and progression of this disease (Jakobsdottir, Gorin, Conley, Ferrell, & Weeks, 2009). While additional genes may be only minor players in terms of their contribution to the total genetic variance of AMD, effect size does not always correlate with the importance to pathogenesis of AMD. Additionally, because other loci do exist which have yet to be elucidated (Abecasis et al., 2004; Fisher et al., 2005; Iyengar et al., 2004; Jun et al., 2005; Kenealy et al., 2004; Majewski et al., 2003; Schick et al., 2003; Schmidt et al., 2004; Seddon, Santangelo, Book, Chong, & Cote, 2003), it is clear that the percent of genetic variance is not proportional to understanding the pathophysiology of disease or understanding gene–gene interactions. It may therefore be important to identify and characterize additional risk factors that may augment the value of known risk factors as prognostic tools in order to identify individuals that require closer follow-up and early intervention (Jakobsdottir et al., 2009; Ware, 2006). Moreover, it is equally important to determine the mechanism of disease, not just risk factors, so that appropriate avenues for treatment may be identified and explored.

Retinoic acid receptor-related orphan receptor alpha (*RORA*) is one of three retinoid-related orphan receptors that compose a distinct subfamily of nuclear receptors (Hubbard et al., 2009). *RORA* is known to play a key role in the regulation of circadian rhythms, the development of cones, bone morphogenesis, angiogenesis, and pathways including immunity/inflammation, lipid metabolism, and cholesterol (Besnard et al., 2001, 2002; Boukhtouche, Mariani, & Tedgui, 2004; Boukhtouche et al., 2006; Lau et al., 2008; Zhu, McAvoy, Kuhn, & Smith, 2006).

In vitro studies have identified cholesterol as a natural ligand of *RORA* (Kallen et al., 2002). In addition to binding cholesterol, *RORA* has also been shown to regulate lipoproteins, such as high density lipoprotein, serum amyloid A, and apolipoprotein A1 (Lau et al., 2008; Migita, Satozawa, Lin, Morser, & Kawai, 2004; Voyiaziakis et al., 1998). Further evidence for the role of *RORA* in cholesterol (“cholesteROR”) metabolism comes from phenotypic examination of the *RORA* deficient *staggerer* mouse (*RORA*^{sg}) that displays an increased susceptibility to arteriosclerosis and dislipidemia (Boukhtouche et al., 2004; Jetten & Ueda, 2002; Kopmels, Mariani, Taupin, Delhaye-Bouchaud, & Wollman, 1991; Lau et al., 2008; Mamontova et al., 1998).

If cholesterol/lipid transport and metabolism are involved in the pathophysiology of neovascular AMD, then genes that are intrinsic to these pathways may be differentially expressed between patients with neovascular AMD and their unaffected siblings. In order to identify novel candidate genes and pathways with biological relevance to AMD pathophysiology, we performed linkage analysis and gene expression microarray analysis on extremely discordant sibling pairs. An “extreme” sibling pair consists of one sibling with a trait value in the top 10% of disease severity and the other sibling with a trait value in the bottom 10% of disease severity (Risch & Zhang, 1995, 1996). Based on the results of these studies and biological plausibility in AMD etiology, the candidate gene *RORA* was chosen for further analysis. Any significant haplotypes identified in the family-based cohort of European descent were then tested in an unrelated case-control cohort from Central Greece.

2. Methods

2.1. Family patient population

The protocol was reviewed and approved by the Institutional Review Boards at Massachusetts Eye and Ear Infirmary, Boston, Massachusetts and conforms to the tenets of the Declaration of Helsinki. Eligible patients were enrolled in this study after they gave informed consent, either in person, over the phone, or through the mail, before completing a standardized questionnaire and donating 10–50 ml of venous blood.

Details for the recruitment of the 196 sibling pairs, comprised mainly of individuals of European ancestry, are described elsewhere (DeAngelis et al., 2008) (Population characteristics are summarized in Supplementary Table 1). In brief, all index patients were aged 50 years or older, except where one individual was 49 years of age, and had the neovascular form of AMD in at least one eye, defined by subretinal hemorrhage, fibrosis, or fluorescein angiographic presence of neovascularization documented at the time of, or prior to, enrollment in the study. Patients whose only exudative finding was a retinal pigment epithelium detachment were excluded because this finding may not represent definite neovascular AMD. Patients with signs of pathologic myopia, presumed ocular histoplasmosis syndrome, angioid streaks, choroidal rupture, any hereditary retinal diseases other than AMD, and previous laser treatment due to retinal conditions other than AMD were also excluded.

Of the 196 sibling pairs, 150 were extremely phenotypically discordant. That is pairs where the unaffected siblings had normal maculae at an age older than that at which the index patient was first diagnosed with neovascular AMD. Normal maculae (defined as the zone centered at the foveola and extending two disk diameters, or 3000 μm, in radius) fulfilled the following criteria: 0–5 small drusen (all less than 63 μm in diameter), no pigment abnormalities, no geographic atrophy, and no neovascularization [as defined previously; AMD “category 1 or less” on the Age-Related Eye Disease Study (AREDS) scale (AREDS Research Group, 2000)]. Disease status of every participant was confirmed by at least two of the investigators by evaluation of fundus photographs or fluorescein angiograms except when one of the investigators directly examined an unaffected sibling during a home visit ($n = 4$ cases). Smoking data, as measured in pack years, was available for every participant.

An additional 46 discordant sibling pairs were analyzed where each pair was comprised of one sibling (the index sibling) with neovascular AMD and the other sibling (the control sibling) with mild or very early AMD [AREDS category 2 (AREDS Research Group, 2000)] at 65 years of age or older in most cases. Siblings were categorized as early AMD only if they met the following criteria for the definition of AREDS category 2: small (<63 μm) drusen with total area $\geqslant$125 μm diameter circle, or at least one intermediate