Published in final edited form as:

Ophthalmic Genet. 2006 December ; 27(4): 151–155. doi:10.1080/13816810600976871.

Mutation Screen of the Cone-Specific Gene, *CLUL1*, in 376 Patients with Age-Related Macular Degeneration

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Abstract

Clusterin is a secreted glycoprotein expressed ubiquitously in many tissues that appears to function as a molecular chaperone capable of protecting stressed proteins. It is upregulated in many different forms of neurodegeneration and is thought to represent a defense response against neuronal damage. Clusterin has been found to be a common protein identified in drusen preparations isolated from the retina of donor eyes of patients with age-related macular degeneration (AMD), the leading cause of blindness in the elderly population of developed countries. A retina-specific clusterin-like protein (CLUL1) showing nearly 25% identity to clusterin at the protein level was recently cloned and shown to be expressed specifically in cone photoreceptor cells. For these reasons, we investigated *CLUL1* as a candidate gene for AMD. A mutation screen of the entire coding region of the *CLUL1* gene in 376 unrelated patients with AMD uncovered three sequence variations, one isocoding change and two intronic changes. One intronic change appears significantly less frequent in patients with the more severe forms of AMD than in control subjects, suggesting that this variant may reduce the risk for AMD or may be linked to a nearby variant that may reduce AMD risk. Variant alleles of the *CLUL1* gene were found; however, none are considered pathogenic. None of the variants identified are predicted to create or destroy splice donor or acceptor sites based on splice-site prediction software.

Keywords

Retinal degeneration; age-related macular degeneration; clusterin; mutation

INTRODUCTION

Clusterin (CLU) is widely expressed in many tissues and appears to be involved in a wide range of processes, including lipid transport, regulation of the complement cascade, membrane recycling, apoptosis, and cytoprotection of cellular membranes.¹⁻³ Most recently, CLU has been shown to function as a molecular chaperone.⁴ Evidence for several mechanisms of neuroprotection by CLU have been described including: 1) functioning as an anti-apoptotic signal, 2) protecting against oxidative stress, 3) inhibiting the membrane attack complex of complement proteins activated as a result of inflammation, and 4) binding to hydrophobic regions of stressed proteins.⁵

CLU is expressed in a broad range of eye tissues, including the retinal pigment epithelium, photoreceptor cells, and ganglion cells.⁶ CLU is found in these different eye tissues in several distinct isoforms, that are likely to reflect the different post-translational modifications that allow CLU to be involved in different cellular processes.⁷ Studies have shown that CLU expression is upregulated in neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease and also in retinal diseases such as retinitis pigmentosa and several mouse models of retinal degeneration, indicating an important role in neural degeneration.⁸⁻¹¹

Age-related macular degeneration (AMD) is the leading cause of irreversible visual loss in the elderly Western population over the age of 50 years. AMD is a slow, progressive disease that typically starts with a few macular drusen, the extracellular deposits considered a major risk factor for developing the disease, but evolves into many drusen and can lead to geographic atrophy (GA), choroidal neovascularization (CNV), and irreversible vision loss

in the later stages. Substantial evidence indicates that AMD has a genetic component and genetic variants within the complement system have been identified that appear to confer increased susceptibility or protection from AMD development.^{12–16}

Proteomic analyses of drusen isolated from donor eyes show that CLU is one of the most commonly encountered proteins in drusen isolated from AMD patients.¹⁷ A retina-specific CLU-like protein (CLUL1) was recently cloned and shown to be expressed specifically in postnatal cone photoreceptor cells.¹⁸ CLUL1 shows light-dependent translocation, being located to the outer segment in the light and to the synaptic terminal in the dark, indicating that CLUL1 is likely to be critical for normal cone function.¹⁹ Furthermore, CLUL1 transcripts are developmentally regulated in parallel with retinal differentiation, suggesting a functional role during photoreceptor differentiation.¹⁸ In addition, *cis*-elements of CRX (cone-rod homeobox) binding sites and PCE-1 (photoreceptor conserved element 1) have been identified in CLUL1 promoter regions, indicating that CLUL1 is a cone photoreceptor-specific gene under CRX regulation.¹⁹ Based on its developmental regulation, distinct localization, and possible involvement in a wide range of cellular processes similar to CLU, we considered *CLUL1* to be a candidate gene for AMD. We report here our analysis of this gene in 376 patients with AMD.

MATERIALS AND METHODS

Ascertainment of Patients

This study conformed to the tenets of the Declaration of Helsinki and was approved by the Internal Review Boards of the Cleveland Clinic Foundation and the Louis Stokes Cleveland Department of Veterans Affairs Medical Center. All blood samples were obtained after informed consent was secured. We collected a total of 376 index cases with AMD that were diagnosed through ophthalmologic examination and subcategorized into one of four experimental groups using the criteria defined by the Age-Related Eye Disease Study.²⁰ Of the 376 patients, 33 were defined as Category 1 AMD, 92 were Category 2, 47 were Category 3, and 204 were Category 4. Of these patients, 369 were over the age of 60 years; the remaining seven were over the age of 50 years. The age range was between 52 and 97 years, with a mean age of 77.5 years. A total of 191 unrelated individuals without a family history of retinal disease were used as normal control subjects. Of these, 176 were over the age of 60 years; the remaining 15 were over the age of 50 years. The age range of this group was between 50 and 92 years, with a mean age of 72.3 years. Leukocyte nuclei were prepared from blood samples, followed by DNA purification using standard protocols.

Mutation Screening

For mutation detection, polymerase chain reaction (PCR) products corresponding to the complete known *CLUL1* coding sequence (GenBank RefSeq NM_014410) were amplified from genomic DNA and analyzed by the single-strand conformation polymorphism (SSCP) technique. Nine primer pairs were designed to cover the eight coding exons as well as the immediately flanking intron sequences. They are listed in Table 1 along with the PCR conditions. Two primer pairs were designed for exon 5 in order to split the exon into two fragments to yield smaller-sized amplicons for SSCP. In addition, the amplified DNA fragments encompassing exon 5b were digested with the restriction endonuclease *MseI* to yield smaller fragments for SSCP analysis. Exons 7 and 8 were also digested with the restriction endonucleases, *HinfI* and *MboI*, respectively, to yield smaller fragments. The buffer pH, Mg^{++} concentration, annealing temperature, and presence or absence of 10% dimethyl sulfoxide were tailored to each primer pair to yield optimal amplification.

Variant bands detected by SSCP were analyzed by sequencing the corresponding PCR-amplified DNA segments using the Quick Start sequencing kit (Beckman-Coulter, Fullerton, CA, USA) following the manufacturer's protocol. A CEQ-2000 automated sequencer (Beckman-Coulter) was used to resolve sequences.

RESULTS

Three sequence changes (IVS2 + 18C → T, Leu5, and IVS8 + 10insTGCCTGGTTAGGAA) were identified in *CLUL1* (Table 2). None of these changes appear likely to alter the sequence of the encoded protein.

The IVS2 + 18C → T intronic change had a minor allele frequency of 31.8% in patients and 37.0% in age-matched normal controls (Table 2). This change appears to be a common variant and is present in the NCBI SNP database (rs518471). Across all patient groups, there was no significant difference between patients and controls (Fisher's exact test; $p = 0.083$). When only the more numerous Category 4 patients ($n = 204$) were considered the minor allele frequency of this variant ($119/408 = 29.2\%$) was significantly different ($p = 0.023$) compared to controls ($140/378 = 37.0\%$). For both the patient and control populations, there was no significant difference between the observed genotype frequencies with those expected under Hardy-Weinberg equilibrium ($\chi^2 = 2.23$, $p = 0.33$ in patients; $\chi^2 = 1.73$, $p = 0.42$ in controls).

The isocoding sequence change, Leu5, was identified heterozygously in four Category 4 AMD patients and was not found in normal controls, yielding a minor allele frequency of 0.5% in patients. IVS8 + 10(14-bp ins) was identified heterozygously in one Category 2 AMD patient and two normal controls, all of African-American descent. The minor allele frequency for this sequence change is 0.1% in patients and 0.6% in normal controls.

None of the isocoding or intronic changes described is predicted to create or destroy splice donor or acceptor sites based on splice-site prediction software available at the neural network website (http://www.fruitfly.org/seq_tools/splice.html). No second change was found in the *CLUL1* gene in the five patients identified with the changes Leu5 or IVS8 + 10(14-bp ins).

DISCUSSION

In this study, mutation screening of *CLUL1* in 376 patients with AMD failed to reveal mutations that can be uniquely associated with disease. Only three sequence variations were identified. All three are likely to be nonpathogenic polymorphisms as none alter the predicted wild-type amino-acid sequence. However, the IVS2 + 18C → T intronic change warrants additional consideration. Our data indicate that the T allele is less frequent in patients with the more severe Category 4 grade of AMD compared to normal controls, suggesting that this variant is associated with a reduced risk for developing advanced AMD. Whether this variant is actually involved or is simply linked to a different nearby gene variant remains to be determined. In addressing these issues, additional studies of different and larger populations of patient and control samples will be required.

Our data suggest that sequence variations in *CLUL1* are rare. It is possible, however, that pathogenic mutations in this gene might exist outside the coding exons and flanking intron splice sites screened in this study. It is also possible that pathogenic mutations occur in *CLUL1* in a form of retinal degeneration not included in our patient set. Nonetheless, our results indicate that *CLUL1* mutations are not causally associated with AMD.

Acknowledgments

Supported by Karl Kirschgessner Foundation, American Health Assistance Foundation, Foundation Fighting Blindness, the Medical Research Service, Department of Veterans Affairs, R24 EY15638, Research to Prevent Blindness, and the State of Ohio (BRTT grant).

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TABLE 1

PCR conditions and primers used to amplify *CLUL1* in this study.

Exon	Sense primer	Antisense primer	Anneal temp.	Buffer (pH)	MgCl ₂ (mM)	10% DMSO	Fragment (bp)
2	caaatggaaagacatttgatgcgggg	aacatgcaaacagagacacagcaca	54	8.6	1.5	No	204
3	catgaaagggtgggaactgggg	gtccagatctgtgtaagaaacagac	50	8.4	1.5	Yes	267
4	agggtcacatagatcatgaaatgg	gggtcccttaagaagactgtgaaatct	52	8.4	1.5	Yes	268
5a	ttatccattctgtccctactc	ggctcgtatctgatatgaaatgtg	50	8.4	1.5	No	278
5b	ttatccattctgtccctactc	gaaacgtatatacaatgactcag	50	8.4	1.5	Yes	364
6	gcaaacctctatgacactaacgtg	ctatttcttttgggcaccgaaac	50	8.4	1.5	Yes	269
7	cccaccctcaagtttcatggactt	caaggatagcacggtcatttcgg	58	8.4	1.5	Yes	322
8	agcctatccctgactaagggtggtat	tccgtcaacaaggcattcctaac	52	8.6	1.5	No	316
9	gggtattctattaccatcctctggac	ccagggtcccgatgagagataat	54	8.4	1.5	No	169

Sequence alterations identified in *CLUL1*.

TABLE 2

Exon	DNA change	Sequence change	Protein change	Allele frequency AMD patients	Allele frequency normals
2	IVS2+18C → T	tcttaccctgig → cttatctctgig		239/752 = 31.78%	140/378 = 37.04%
2	C15G	CTC → CTG	Leu5Leu	4/752 = 0.53%	0/382 = 0.00%
8	IVS8 + 10insTGCCTGGTTAGGAA	agagtgcc → agagtgccctggtaggaatgcc		1/752 = 0.13%	2/360 = 0.56%