Mitochondrial DNA Haplogroups Associated with Age-Related Macular Degeneration

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Purpose. To examine the mtDNA control regions in normal and age-related macular degeneration (AMD) retinas. To identify the mtDNA variations associated with AMD.

Methods. Retinas from 10 normal and 11 AMD globes were isolated and analyzed for mtDNA rearrangements by long extension-polymerase chain reaction (LX-PCR) and for the nature and frequency of single-nucleotide polymorphisms (SNPs) in the mtDNA control region by direct sequencing. Blood DNA was extracted from 99 AMD and 92 age-matched control subjects. The sequence variations that define haplogroups H, I, J, K, T, V, X, and U were characterized by PCR, restriction enzyme digestion, and/or sequencing.

Results. LX-PCR of retinal mtDNAs revealed high levels of rearrangements in the patients with AMD and the control subjects, consistent with the decline in mitochondrial function with age. However, the AMD retinas had higher oxidized DNA levels and a higher number of SNPs than controls ($P = 0.02$). The control region SNPs T16126C and A73G, commonly found in haplogroups J and T, were more frequent in the AMD retinas than in normal retinas. The associations between AMD and haplogroups J and T were confirmed and extended by analysis of blood DNA. SNPs at position a T16126C (J; odds ratio [OR] = 3.66), T16126C+G13368A (JT; OR = 10.27), A4917G+A73G (T4; OR = 5), and T3197C+A12308G (U5; OR = ∞), were all strongly associated with AMD.

Conclusions. AMD retinas exhibited increased mtDNA control region SNPs compared to normal retinas. This correlated with an increased frequency of mtDNA SNPs associated with haplogroups J, T and U in patients with AMD. These results implicate mitochondrial alterations in the etiology of AMD. (*Invest Ophthalmol Vis Sci.* 2009;50:2966–2974) DOI:10.1167/iovs.08-2646

Age-related macular degeneration (AMD) is a major cause of blindness among the elderly in the developed world. Approximately 23% of individuals 65 to 74 years of age and 35% to 40% of those 75 to 84 years of age have some pathologic signs of AMD. Studies have implicated genetic factors, inflammation, and oxidative stress in the development of AMD.[1–14]

Organs requiring large amounts of energy, such as brain, heart, muscles, and retina, are dramatically affected by mitochondrial dysfunction. The mitochondrial (mt)DNA defects associated with diseases can be inherited maternally, such as Leber's hereditary optic neuropathy, or develop through somatic mutations. Generally, the mtDNA defects are either point mutations or single nucleotide polymorphisms (SNPs) or alternatively, large scale deletions or rearrangements such as that found in chronic progressive external ophthalmoplegia and Kearns-Sayre syndrome. Chiefly responsible for aerobic respiration, mitochondria are also used in some apoptotic pathways[15] that are thought to be involved in the pathologic course of AMD.[16]

The maternally inherited mtDNA is 16,569 nucleotide (nt) pairs and encodes for 37 genes, 13 protein subunits essential for oxidative phosphorylation (OXPHOS), 2 ribosomal RNAs (rRNA), and 22 transfer RNAs (tRNA).[17,18] mtDNA is susceptible to oxidative damage and has a higher sequence evolution rate than does the nuclear DNA. Over the past 200,000 years, mtDNA variations have accumulated along radiating maternal lineages, and individual mtDNA haplogroup tree branches correlate with the geographic origin of the populations in which they are found. Many haplogroups are founded by one or more functional genetic variants and associated SNPs, which may modify mitochondrial OXPHOS to facilitate the adaptation of the mitochondrial energetics of that population to its environment (e,g., nature and availability of calories, thermal stress, infectious agents) Haplogroups are increasingly being correlated to a broad spectrum of common diseases.[16,19–28] Most recently, an association of mtDNA haplogroups with characteristic AMD retinal lesions was demonstrated.[29] Moreover, A4917G, a defining polymorphism for the T haplogroup, was reported as an independent predictor of AMD.[30]

The mtDNA control region is noncoding but important for mtDNA replication and transcription. The H- and L-strand promoters, sites for origins of replication, mitochondrial transcription factor A binding sites, and conserved sequence blocks are located in the mtDNA control region. Mutations in the control region have been described in patients with Alzheimer's disease and other disorders associated with oxidative stress.[31]

Using retinal DNA in the present study, we sequenced the entire mtDNA control region from AMD and normal retinas to identify variations. Our results showed that AMD retinas had increased numbers of SNPs per individual in the control region compared with normal retinas. Then, using informative SNPs,

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we analyzed blood DNA to establish the haplogroups for each individual in the study and showed that AMD was associated with mtDNA polymorphisms characteristic for the J, T, and U haplogroups.

Materials and Methods

Collection of AMD and Age-Matched Control Retinas

Normal retinas ($n = 10$; mean age, 80 ± 4.33 years; range, 52–97) and AMD retinas ($n = 11$, mean age 81.4 ± 2.65 years; range, 69–93) were collected from the National Disease Research Interchange (Philadelphia, PA) and the San Diego Eye Bank (Table 1). The retinas were dissected, snap frozen and stored at −70°C. The diseased specimens had a clinical diagnosis and medical history of AMD that significantly impaired vision. A board-certified ophthalmologist using a dissecting microscope verified the pathology in the macular area.

Long-Extension Polymerase Chain Reaction (LX-PCR)

The total DNA isolated from normal and AMD retinas were used for LX-PCR amplification by modifications of methods described previously.[32–34] LX-PCR products were separated by electrophoresis on a 0.8% agarose gel stained with ethidium bromide. The mtDNA rearrangements (bands < 16 kb) were quantified (manual counting) by two independent investigators.[33] The method for LX-PCR has been demonstrated to be reliable and represents the mtDNA through verification by Southern blot using a full-length digoxigenin-labeled mtDNA probe by our laboratory as well as others.[34–36] The LX-PCR technique was further substantiated by sequencing individual bands showing they were indeed mtDNA and not artifact.[36]

Immunohistochemistry of AMD and Normal Retinas

Immunohistochemistry was performed on paraffin embedded AMD ($n = 5$) and normal ($n = 5$) eyes using mouse anti-OXPOS complex V subunit *d* monoclonal antibodies (anti-ATP synthase subunit *d*; Invitrogen-Molecular Probes, Inc, Eugene, OR) and goat anti-8-hydroxydeoxyguanidine (8-OH-dG) polyclonal antibody (Chemicon, Temecula, CA). 8-OH-dG recognizes oxidized DNA bases damaged by hydroxyl radicals. Nuclei were stained with DAPI stain (Molecular Probes). Controls samples included use of the secondary antibody alone.

Table 1. Donor Information for Normal and AMD Retinas

Donor	Age (y)	Sex	Ethnicity	Stage
N-3	52	M	C	N/A
N-5	79	M	N/A	N/A
N-6	73	N/A	N/A	N/A
N-7	76	M	C	N/A
N-8	79	F	C	N/A
N-9	79	M	C	N/A
N-10	85	F	C	N/A
N-11	88	F	C	N/A
N-12	91	M	C	N/A
N-13	97	F	C	N/A
A-14	69	F	C	Disciform scar
A-15	88	M	C	Macular atrophy
A-16	77	F	C	Disciform scar
A-17	78	F	C	Macular atrophy
A-19	83	F	C	Disciform scar
A-20	89	F	C	Macular atrophy
A-21	71	F	C	Macular atrophy
A-22	N/A	M	C	Disciform scar
A-23	93	F	C	Disciform scar
A-24	88	F	C	Disciform scar
A-25	90	M	C	Disciform scar

N, normal retinal tissue; A, AMD retinal tissue; N/A, not available; C, Caucasian.

Collection of Blood Specimens from AMD and Control Patient Selections

The study was approved by the institutional review boards of University of California Irvine (HS 2003-3131) and Cedars-Sinai Medical Center (IRB 1708). Informed consents were obtained from participants and the study was performed according to the tenets of the Declaration of Helsinki for research involving human subjects. A total of 154 subjects, including 73 control patients (average age, 76.18 ± 0.7886 years; range, 65–93) and 81 patients with AMD (average age, 77.94 ± 0.7333 years; range, 65–92) were studied ($P = 0.104$). The patients with AMD were from a private practice ophthalmology office that specializes in retinal diseases (Retina-Vitreous Associates Medical Group). The age-matched control patients were from a private practice general ophthalmology office (American Eye Institute). The offices are approximately 2 miles apart and serve the same demographic and ethnic population, which was predominantly European haplogroup Caucasian. In the case population there were 91.4% European haplogroup Caucasians and 8.6% other/non-European lineage. The control population was 86.3% European haplogroup Caucasians and 13.7 other/non-European lineage ($P = 0.124$). Therefore, in this study the cases and controls were matched for both age and ethnicity.

Classification of AMD

The subjects underwent a complete dilated ophthalmic examination by Board certified ophthalmologists (DSB, ABN, and MCK) including slit lamp and indirect ophthalmic examinations with a 90-D lens or a fundus contact lens. Fundus photos, fluorescence and/or indocyanine green angiography were performed. The photographs and angiograms were read by masked graders who were board certified retinal specialists.[8] The subjects were graded according to the Clinical Age-Related Maculopathy Staging System (CARMS).[37] Grade 3 had large soft drusen or several intermediate size drusen or drusenoid retinal pigment epithelial detachment and for this study is referred to as early AMD (see Table 5). In this study, the late-term AMD is the combination of grade 4, which is geographic atrophy, and grade 5, which is neovascular or serous exudative AMD. No stage 1 or 2 patients with AMD were included in this study.

Extraction of Total DNA from Blood and Retinal Tissue

DNA was extracted from venous blood samples of case and age-matched control populations (Puregene DNA extraction kit; Gentra, Minneapolis, MN). DNA was extracted from frozen retinal tissue by methods described previously.[34] In this study we used blood DNA for the haplogroup studies and the retinal DNA for the LX-PCR amplification and sequencing of the mtDNA control region. This is consistent with numerous investigations that use blood DNA to identify haplogroup patterns of large groups of people for epidemiologic, disease profiles, and population migration studies.

Haplogroup Analyses

PCR and restriction enzyme digestions on the mtDNA were performed to ascertain the mitochondrial haplogroup of each patient.[22,24,38] Table 2 presents the oligonucleotide primers, polymorphic sites, PCR parameters, and restriction enzymes used. Results were visualized on 2% agarose gels. We have defined haplogroup UK as positive for the A12308G SNP, K as positive for both the A12308G and G9055A SNPs, and U as positive for haplogroup A12308G SNP but negative for the A9055G SNP. The T haplogroup is defined by G13368A and A4917G. The J haplogroup is defined by G13708A, C16069T, and T16126C (see Table 4).

Sequencing

All sequenced PCR products were treated to remove unused primers and nucleotides (ExoSAP-IT; USB Corp., Cleveland, OH). Retina prod-

TABLE 2. mtDNA Haplogroup Analysis

Related Haplogroup	Primer	Restriction Enzyme
H	F AATGATCTGCTGCAGTGCTC	*Alu*I
7028	R TCCGGATAGGCCGAGAA	
I	F GCTATCGGGCCCATACCCCGAAAATGT	*Bts*I
4529	R GGATGCGGTTGCTTGCGTGAGGAAATA	
J	F CCGGAGATGAAAACCTTTTTCCAAGGAC	*Hpy*CH4V
16126	R TGATGTGTGATAGTTGAGGGTTGATTGC	
J	F CCGGAGATGAAAACCTTTTTCCAAGGAC	*Afl*II
16069	R ATGTACGAAATACATAGCGGTTGTTGATGCTT	
J	F TCCATCATCCACAACCTTAAC	*Bst*NI
13708	R GATTGTTAGCGGTGTGGTCG	
K	F ACCACCCAACAATGACTAATC	*Hae*II
9055	R GTTGTCGTGCAGGTAGAGG	
T	F TAGCCTTCTCCACTTCAAGTC	*Sau*96I
13368	R AGAAACCTGTGAGGAAAGGTATT	
V	F ACCTATCACACCCCATCCTAAA	*Nhe*I
4580	R AAGGATTATGGATGCGGTT	
UK	F GCCACATAGCCCTCGTAGT	*Hin*fI
12308	R TATTTGGAGTTGCACCAAGATT	
X	F ACTTAACTTGACCGCTCTGAG	*Dde*I
1715	R ATTGGTGGCTGCTTTTAGGC	
T	F CTCCGGGCCCATAACACTTGGGGGTAG	*Apa*LI
73	R TGTGTGGAAAGTGGCTGTGCAGACATTCA	
T	F GCCCCCATCTCAATCATATACCAAATCTCTCC	*Bfa*I
4917	R TGCGAGATAGTAGTAGGGTCGTGGTGCTGGA	

ucts were sequenced for the mtDNA control region (F: CTAAGCCAATCACTTTATTG; R: GCTGCGTGCTTGATGCTTGT). Haplogroup products were sequenced for specific SNPs. Sequencing was performed at the UCLA Sequencing and Genotyping Core facility. Sequence analyses were with the Mutation Surveyor program, (Softgenetics, State College, PA). Base changes were called heteroplasmic if the sequencing results gave a reduction and simultaneous increase of the SNP allele of more than ±30%. Homoplasmy was identified when there was no change in the signal peak heights of ±20%. Primers were designed using PRIMER 3 (Whitehead Institute for Biomedical Research; MIT, Cambridge, MA).[39]

Statistical Analyses

LX-PCR and semiquantitative PCR were analyzed by unpaired *t*-test (two-tailed) using commercial software (GraphPad Prism; GraphPad Software, San Diego, CA) while haplogroup study data were analyzed by the Fisher exact text. Odds ratios and *P* values were calculated at http://home.clara.net/sisa/*t*-test.htm.

RESULTS

mtDNA Analyses of Retinal Tissues

The mtDNA isolated from AMD ($n = 11$) and age-matched control ($n = 10$) retinas were examined using LX-PCR analyses, which allows amplification of the entire mitochondrial genome and any subgenomic-sized product that contains the primer sites. The full-length mtDNA LX-PCR product is ~16.2 kb. The smaller sized mtDNA bands which represent rearrangements, degradations or deletion, were quantified and the mean ± SEM calculated (Fig. 1). The normal versus AMD retinal LX-PCR mtDNA had 9.60 ± 1.24 and 10.89 ± 0.82 smaller sized bands per individual, respectively, ($P = 0.41$). The smaller products were heterogeneous in size and no one pattern was dominant. The ratios of mtND2, representing mtDNA, and 18s RNA, representing nDNA, in the normal and AMD retinal samples were similar to each other (1.34 ± 0.27 vs. 1.371 ± 0.489,$P = 0.867$; data not shown). These results show that the retinas of

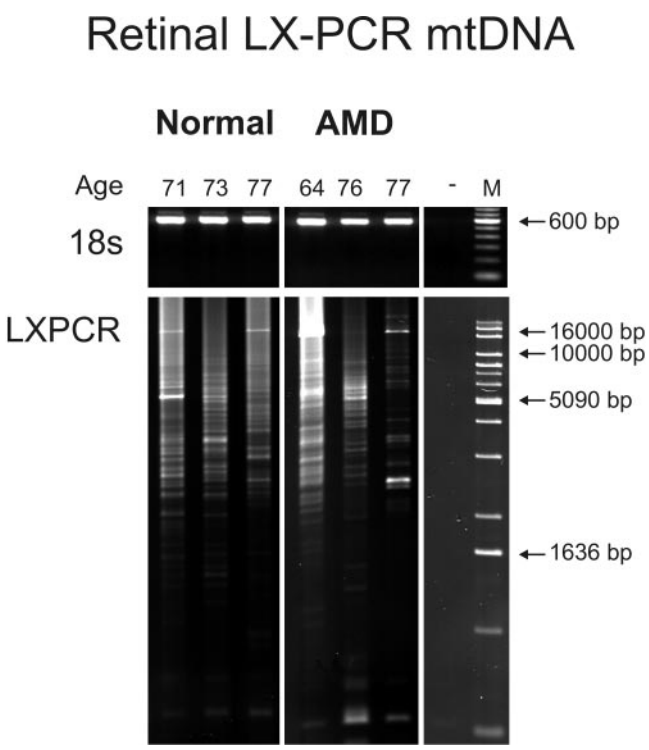

FIGURE 1. LX-PCR of retinal mtDNA. Representative ethidium bromide–stained gel showing LX-PCR mtDNA from AMD and control retinas. The ages are given above each lane representing a different individual. The 16.2-kb product corresponds to the full-length mtDNA and the smaller sized LX-PCR products (<16.2 and >1.0 kb) represent mtDNA rearrangement/deletions. The 18s represents nuclear DNA. Quantitation of the number of smaller sized bands per individual in AMD and control retinas showed a similar number of smaller products (10.89 ± 0.82 vs. 9.60 ± 1.24 smaller sized bands per individual). M, marker.

80 year olds, whether AMD or control, have sustained a high level of somatic mtDNA damage, which is likely to have resulted in a significant age-related decline in mitochondrial function.

Evidence of Oxidative Damage in AMD Retinas

To determine whether this high level of mtDNA damage is due to oxidative stress, we assessed the relative levels of oxidative DNA damage by staining the retinas with 8-OH-dG, a sensitive and stable marker of oxidatively damaged DNA. The retinal structure was defined by DAPI staining for the nuclear layers and by staining for the nuclear DNA-encoded mitochondrial subunit ATP synthase subunit *d*. In Figure 2 the photoreceptor nuclei stained blue, the ATP synthase subunit *d* stained green, and the 8-OH-dG rich DNA stained red. The ATP synthase subunit *d* is found in the retinal outer segment adjacent to the photoreceptor nuclei of the normal and AMD retinas. In areas of active disease, the AMD retinas show increased staining of 8-OH-gD in the outer segment region, suggesting increased oxidative damage to the mtDNA. Age-matched normal retinas had minimal 8-OH-dG staining of the retina.

Sequencing Analyses of the Retinal mtDNA Control Region

Increased mtDNA oxidative damage could increase the mtDNA somatic mutation rate. To assess this possibility, the entire mtDNA control region of the retinas was sequenced. A total of 50 nucleotide variants were detected in the 21 individuals studied, with each individual having between 1 to 12 SNPs (Tables 3A, 3B). On average, AMD retinas were found to have 8.9 ± 0.96 nucleotide changes while controls retinas had 5.8 ± 0.71 variants ($P = 0.02$). Thus, the AMD retinas were found to have more mtDNA control region variants than normals, perhaps reflecting a greater somatic mtDNA mutation rate.

Haplogroup Analyses Case and Control Populations

Analysis of the retinal mtDNA control region SNPs revealed that specific variations correlated with AMD. The A73G SNP, a common mtDNA variation, was found in 91% of AMD retinas (10/11) but in 40% (4/10) of the normal retinas (Table 3C), the T16126C SNP was present in 64% (7/11) of AMD retinas compared with 20% (2/10) of control retinas and the C16069T SNP was found in 45% (5/11) of AMD retinas versus 10%(1/10) of control retinas. The T16126C and C16069T SNPs are commonly associated with mtDNA haplogroups J and the A73G SNP is frequently associated with a subset of haplogroup T.

Since the number of retinal samples studied were small and to determine whether this correlation was statistically significant, we examined the peripheral blood samples from 81 case patients and 73 age-matched controls to investigate association with common European mtDNA haplogroups H, I, J, K, T, U, V and X. Table 4 shows the distribution of SNPs from the subjects with any AMD (early and late AMD combined). The haplogroup H was present in 33% of the AMD and 32.9% of control subjects (Table 4) which is consistent with previous studies of European populations and demonstrates the similarity of our case and control population substructure. No statistically significant differences were seen for haplogroups H, I, K, V, or X. Consistent with the retinal study we found a strong association of T16126C SNP in 24.7% (20/81) of the any AMD population compared to 8.2% (6/73) of the controls (odds ratio [OR] = 3.66; $P = 0.004$). The G13708A SNP (J haplogroup) had 12.3% (10/81) in the case population and 4.1% (3/73) in the control population (OR = 3.29; $P = 0.044$). The C16069T SNP was present in 9.9% (8/81) of the AMD but 4.1% (2/73) of the control (OR = 3.89; $P = 0.055$) subjects. We also examined combinations of haplogroup-related SNPs (Table 4). The 16126+13368 SNPs (JT haplogroup) had significant association to AMD (OR = 10.27; $P = 0.003$). The case population was analyzed further for the J haplogroup subsets (Fig. 3) and showed J(2/11), J2(3/11), J1A(1/11), and J1C(5/11).

The aggregate T haplogroup SNPs A4917G, G13368A, and A73G SNPs also correlated with AMD. The A4917G SNP was found in 12.2% of the AMD but only 2.7% of the control population (OR = 5; $P = 0.02$). The T4 subset (SNPs 4917+73) also had a significant association with the case population (OR = 5; $P = 0.02$; Fig. 3, Table 4). We also found that the

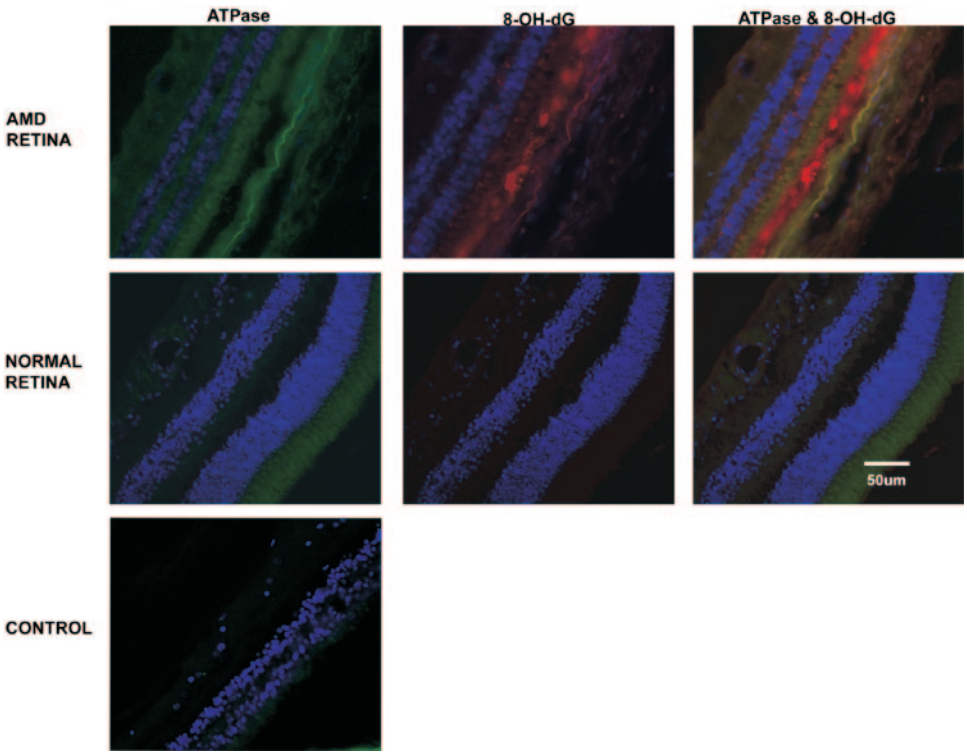

FIGURE 2. Immunohistochemistry shows increased 8-OH-dG staining in AMD retinas (*red*). In normal and AMD retinas, the complex V, subunit d (ATP) staining patterns were similar to each other (*green*). Nuclei are *blue*, as identified with the DAPI staining. In the control tissues, no staining was observed when only secondary antibody (IgG only) was used on the tissue sections.

TABLE 3. Sequencing of the Retinal mtDNA Control Region

A. First 25 Polymorphisms

	Polymorphisms																								
Donor	73 A>G	143 G>A	146 T>C	150 C>T	152 T>C	185 G>A	195 T>C	199 T>C	203 G>A	204 T>C	217 T>C	228 G>A	250 T>C	295 C>T	462 C>T	477 T>C	482 T>C	489 T>C	497 C>T	508 A>G	709 G>A	15833 C>T	15852 T>C	15907 A>G	15924 A>G
N-3	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N-5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
N-6	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	1
N-7	1	0	0	0	0	1	0	0	0	0	0	1	0	1	1	0	1	1	0	0	0	0	0	0	0
N-8	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N-9	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
N-10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N-11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
N-12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N-13	0	1	0	0	0	0	1	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A-14	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
A-15	1	0	0	0	0	1	0	0	0	0	0	1	0	1	1	0	0	1	0	0	0	0	0	0	0
A-16	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A-17	1	0	0	0	0	1	0	0	0	0	0	1	0	1	1	0	0	1	0	0	0	0	0	0	0
A-19	1	0	0	0	0	1	0	0	0	0	0	1	0	1	1	0	0	1	0	0	0	0	0	0	0
A-20	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
A-21	1	0	0	0	0	1	0	0	0	0	0	1	0	1	1	0	1	1	0	0	0	0	0	0	0
A-22	1	0	0	0	0	1	0	0	0	0	0	1	0	1	1	0	1	1	0	0	1	0	0	0	0
A-23	1	0	0	0	0	0	0	1	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1
A-24	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A-25	1	0	0	0	1	0	1	1	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	1	0

B. Remaining 25 Polymorphisms

	Polymorphisms																								
Donor	15928 G>A	16051 A>G	16069 C>T	16092 T>C	16093 T>C	16126 T>C	16129 G>A	16129 G>C	16172 T>C	16182 A>C	16183 Adel	16189 T>C	16192 C>T	16223 C>T	16224 T>C	16239 C>T	16261 C>T	16263 T>C	16294 C>T	16296 C>T	16304 T>C	16311 T>C	16319 G>A	16391 G>A	16519 T>C
N-3	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0
N-5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
N-6	0	0	0	0	2	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	1
N-7	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N-8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
N-9	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	1
N-10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0
N-11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1
N-12	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1
N-13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
A-14	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	1
A-15	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A-16	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
A-17	0	0	1	2	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
A-19	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A-20	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	1	1	1	0	0	0	0
A-21	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A-22	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0
A-23	0	0	0	0	0	0	1	0	1	0	0	0	0	1	0	0	0	0	0	0	0	1	0	1	1
A-24	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
A-25	0	1	0	0	0	0	0	1	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0

(*continues*)

TABLE 3 (*continued*). Sequencing of the Retinal mtDNA Control Region

C. Sequence of Normal and AMD Retinas

	73 A>G	**185** G>A	**228** G>A	**295** C>T	**303** C-CC	**303** C-CCC	**462** C>T	**489** T>C	**16069** C>T	**16126** T>C	**16311** T>C	**16519** T>C
Incidence in control subjects	4/10	1/10	1/10	1/10	4/10	4/10	1/10	1/10	1/10	2/10	4/9	4/9
Incidence in AMD	10/11	5/11	5/11	5/11	3/11	3/11	5/11	5/11	5/11	7/11	1/9	1/9
% Controls	40.00	10.00	10.00	10.00	40.00	40.00	10.00	10.00	10.00	20.00	44.00	44.00
% AMD	91.00	45.00	45.00	45.00	27.00	27.00	45.00	45.00	45.00	64.00	11.00	11.00

0, no allele; 1, homozygous SNP; 2, heterozygous SNP. Numbers are bold for emphasis.

SNPs related to the U haplogroup (A12308G+ and G9005A−) had increased association with AMD (OR = 5.58; $P = 0.013$; Table 4). The combination T3197C+A12308G (subset U5) was found in 6 of 81 case subjects but none of the controls (OR = ∞; $P = 0.019$). Figure 3 shows the U subsets to be U1A(1/11), U4A(1/11), U5A(4/11), U5B(3/11), and U6A(2/11) (Fig. 3).

Table 5 shows the association between the three most prevalent mtDNA haplogroups (J, T, and U) in early AMD (grade 3) and late AMD (grades 4 and 5) compared with the control population. In the early AMD population, the U haplogroup SNPs (A12308G+ and G9055A−) (OR = 10.4; $P = 0.01$) and the T haplogroup SNP A4917G (OR = 7.1; $P = 0.05$) were associated with the case population. The late AMD group showed strong association with all SNPs associated with the J, T, and U haplogroups, including both the single SNPs and combination of SNPs.

Sequencing confirmed the RFLP changes and that restriction site variants were due to the expected nucleotide variants.

DISCUSSION

The retina has one of the highest oxygen consumption rates of any tissue in the body, presumably due to continuous demand for mitochondrial ATP for visual function. The continuous, high exposure of light results in photo and oxidative damage to retinal cells. Since mtDNA damage accumulates with age and is associated with increased mitochondrial oxidative stress,[40] it follows that human retinas would accumulate high levels of mtDNA damage by age 80. This finding was confirmed by LX-PCR analysis of retinas of both AMD and control subjects. Indeed the frequency and distribution of mtDNA rearrangements was sufficiently high to saturate the gel, making subtle distinctions between the control subjects and patients with AMD impossible. The high rate of mtDNA damage maybe the product of oxidative damage[41] based on increased levels of 8-OH-dG of the AMD retinas observed with immunohistochemistry. Increased oxidative DNA damage has also been reported in RPE and choroid of aging rodents.[42] The excessive accumulation of age-related somatic mtDNA variations could severely inhibit the mtDNA biosynthetic capacity, inhibit mitochondrial function, and lead to apoptosis.[43]

Consistent with increased mtDNA damage in the AMD retinas, sequencing of the mtDNA control region revealed an increase in the number of variants in AMD retinal mtDNAs versus controls ($P = 0.02$), which may represent sites for hot spots in this critical mtDNA region. Higher levels of SNPs in the mtDNA control region may impair mitochondrial copies and/or the functional efficiency, thereby contributing to decreased energy production and cell death.[43] Because mtDNA control regions were amplified and sequenced in aggregate for each sample, only those new mutations that predominate would be detected. Presumably, the differential would have been greater if somatic variants at low percentages could have been detected.

Sequencing of the control regions from the AMD retinas revealed that certain haplogroup-associated control region SNPs were more prevalent in the patients with AMD than in the control subjects. These included the C16069T and T16126C SNPs commonly associated with haplogroup J and the A73G SNP frequently associated with haplogroup T. The association observed between retinal mtDNA and haplogroups J and T were then confirmed in a survey of the mtDNA haplogroups of blood samples from 81 AMD and 73 control subjects. Consistent with our findings, Canter et al.[30] reported the haplogroup T-associated SNP A4917G as an independent predictor of AMD, and Jones et al.[29] showed some association of J and U haplogroups with clinically recognized AMD retinal lesions. Haplogroup J was associated with large, soft drusen, which are recognized risk factors for the development of wet AMD, and haplogroup U was linked to retinal pigment abnormalities.[44]

TABLE 4. Distribution of SNPs from Any AMD Compared to Age-Matched Control Subjects

SNPs rs Number	Any AMD (%)	Normal (%)	*P*	OR	95% CI	Related Haplogroups
C7028T rs56497622	27/81 (33)	24/73 (32.9)	0.13	1.02	0.52–1.99	H
A4529T rs9701642	1/81 (1.2)	1/73 (1.4)	0.50	0.90	0.06–12.89	I
G13708A rs28359178	10/81 (12.3)	3/73 (4.1)	0.044	3.29	0.87–12.45	J
C16069T NA	8/81 (9.9)	2/73 (4.1)	0.055	3.89	0.79–18.96	J
T16126C NA	20/81 (24.7)	6/73 (8.2)	0.004	3.66	1.38–9.72	J
T16126C, C16069T NA, NA	8/81 (9.9)	2/73 (2.7)	0.055	3.89	0.80–18.95	J
G13708A, C16069T, T16126C rs28359178, NA, NA	8/81 (9.8)	2/73 (2.7)	0.055	3.89	0.79–18.95	J
T16126C, G13368A NA, rs3899498	11/81 (13.6)	1/73 (1.4)	0.003	10.27	1.41–74.95	JT
G13368A rs3899498	10/81 (12.3)	1/73 (1.4)	0.0068	9.20	1.25–67.75	T
A4917G rs28357980	10/81 (12.2)	2/73 (2.7)	0.02	5	1.05–23.64	T
A4917G, G13368A rs28357980, rs3899498	9/81 (11.1)	1/73 (1.4)	0.012	8.17	1.10–60.76	T
A4917G, A73G rs28357980, rs3087742	10/81 (12.3)	2/73 (1.4)	0.02	5	1.03–23.63	T4
A12308G rs2853498	24/81 (29.6)	16/73 (21.9)	0.08	1.5	0.72–3.11	UK
A12308G, G9055A+ rs2853498, rs28348268	13/81 (16.0)	14/73 (19.2)	0.147	0.81	0.85–1.85	K
A12308G, G9055A− rs2853498, rs28358268	11/81 (13.6)	2/73 (2.7)	0.013	5.58	1.19–26.08	U
A12308G, T3197C, C16270T rs2853498, rs2854131, rs2857290	5/81 (6.17)	0/73 (0)	0.037	Infinity	Infinity	U5
G4580A rs28357975	1/81 (1.2)	1/73 (1.4)	0.501	0.90	0.06–12.89	V
C1715T rs28658366	5/81 (6.2)	4/73 (5.5)	0.263	1.135	0.30–4.39	X

95% CI, 95% confidence interval.

Of interest, their study did not find associations with the late stages of AMD.[29] However, it might be a sampling factor since 85% of their patient population had early AMD, and only 15% subjects had late AMD. In our study the AMD patient base came from retina specialists, and over 77% of patients with AMD had the most severe forms of AMD. This population showed significant associations of SNPs with J, T, and U haplogroups. Control region SNPs associated with U haplogroup were not found in the AMD retinas. The discrepancies between increased frequency of U haplogroup SNPs in blood DNA (11/81) versus retinal DNA may be explained by insufficient numbers of AMD retinas ($n = 11$) which leads to limitations of the power for the retinal cases and controls. A larger number of AMD retinas must be analyzed.

Within European populations, haplogroups J, T, and U are often associated with altered disease risk. The presence of

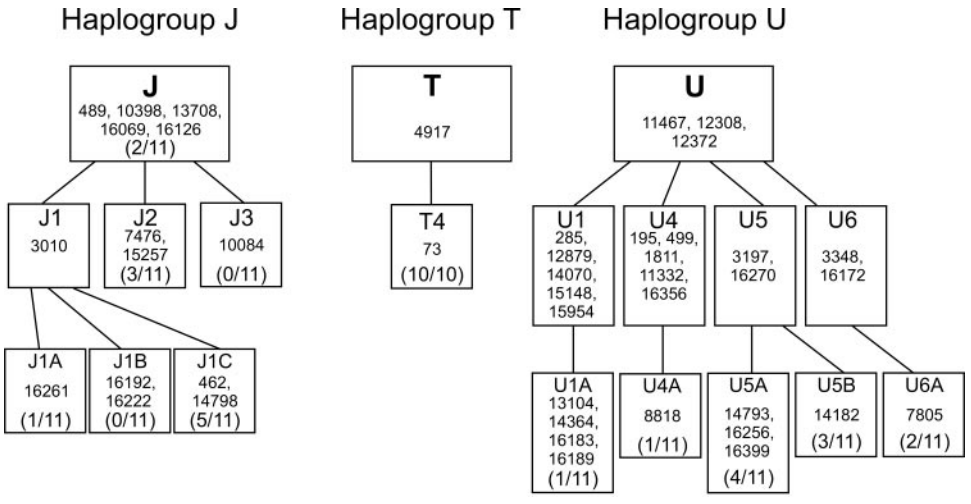

FIGURE 3. Subsets of the J, T, and U haplogroups found in the AMD samples.

TABLE 5. The J, T, and U Haplogroup–Related SNPs of Early and Late AMD Compared to Age-Matched Control Subjects

SNPs	Early AMD	Late AMD	Normal	Related Haplogroups
C7028T, *n* (%)	7/18 (38.8)	20/63 (31.74)	24/73 (32.9)	H
OR [95% CI]	1.29 [0.45–3.77]	0.95 [0.46–1.95]		
P	0.19	0.14		
G13708A, *n* (%)	1/18 (5.5)	9/63 (14.29)	3/73 (4.1)	J
OR [95% CI]	1.52 [0.16–14.39]	3.39 [1.00–15.06]		
P	0.42	0.03		
C16069T, *n* (%)	1/18 (5.5)	7/63 (11.1)	2/73 (4.1)	J
OR [95% CI]	2.31 [0.21–25.13]	4.43 [0.89–22.20]		
P	0.39	0.04		
T16126C, *n* (%)	3/18 (16.67)	17/63 (26.98)	6/73 (8.2)	J
OR [95% CI]	2.23 [0.50–9.96]	4.13 [1.51–11.26]		
P	0.18	0.003		
G13708A, C16069T, T16126C, *n* (%)	1/18 (5.5)	7/63 (11.1)	2/73 (2.7)	J
OR [95% CI]	2.31 [0.21–25.13]	4.44 [0.89–22.20]		
P	0.39	0.04		
T16126C, C16069T, *n* (%)	1/18 (5.5)	7/63 (11.1)	2/73 (2.7)	J
OR [95% CI]	2.31 [0.21–25.13]	4.44 [0.89–22.20]		
P	0.39	0.04		
T16126C, G13368A, *n* (%)	2/18 (11.1)	9/63 (14.3)	1/73 (1.4)	JT
OR [95% CI]	8.17 [0.75–89.07]	10.89 [1.46–81.38]		
P	0.09	0.004		
G13368A, *n* (%)	2/18 (11.1)	8/63 (12.7)	1/73 (1.4)	T
OR [95% CI]	8.17 [0.75–89.07]	9.50 [1.26–71.94]		
P	0.09	0.008		
A4917G, *n* (%)	3/18 (16.67)	7/63 (11.1)	2/73 (2.7)	T
OR [95% CI]	7.1 [1.09–46.25]	4.44 [0.89–22.20]		
P	0.05	0.04		
A4917G, G13368A, *n* (%)	2/18 (11.1)	7/63 (11.1)	1/73 (1.4)	T
OR [95% CI]	8.17 [0.75–89.07]	8.17 [1.06–62.86]		
P	0.09	0.02		
A4917G, A73G, *n* (%)	3/18 (16.67)	7/63 (11.1)	2/73 (1.4)	T4
OR [95% CI]	7.1 [1.09–46.24]	4.44 [0.89–22.20]		
P	0.05	0.04		
A12308G, G9055A, *n* (%)	4/18 (22.2)	7/63 (11.1)	2/73 (2.7)	U
OR [95% CI]	10.4 [1.69–60.85]	4.44 [0.89–22.20]		
P	0.01	0.04		
A12308G, T3197C, *n* (%)	3/18 (16.67)	4/63 (3.17)	0/73 (0)	U5
OR [95% CI]	Infinity [infinity]	Infinity [infinity]		
P	0.006	0.043		

haplogroup J increases the probability of blindness for individuals harboring the milder Leber's hereditary optic neuropathy mutations at positions 11778, 14484, and 10663.[45,46] Haplogroup J has been associated with increased risk of optic neuritis in patients with multiple sclerosis[47]; and haplogroups J, T, and U with altered risk for Parkinson's and Alzheimer's diseases.[19,48,49] It has been proposed that these haplogroup-related SNPs harbor functional polymorphisms that alter the efficiency of mitochondrial energy production[31] by leading to a partially uncoupled OXPHOS which then requires that more calories be consumed for the same amount of ATP produced, resulting in greater body heat production. This effect would be advantageous in the temperate zone where coping with periods of extreme cold stress would be critical for survival.[27,28] Consistent with this model, the sperm motility of haplogroup T and U mtDNAs was less than the common European haplogroup H, and these putative "uncoupled" mtDNAs are enriched in Northern Europe over Southern Europe.[50]

Overall, our results suggest that defects in mitochondrial energy metabolism are important factors in the etiology of AMD. The mtDNA haplogroups associated with reduced mitochondrial ATP production increase the risk of AMD. We hypothesize that the partial and thus subclinical deficiencies in OXPHOS associated with haplogroups J, T, and U are then exacerbated by the age-related accumulation of somatic mtDNA damage, including both mtDNA control region base substitution mutations and total mtDNA rearrangements/mutations. As OXPHOS is inhibited, there is increased production of mitochondrial reactive oxygen species which causes addition mtDNA damage and dysfunction. Ultimately, as mitochondrial energy production falls below the retinal photoreceptor cell bioenergetic threshold, the mitochondrial permeability transition pore (mtPTP) is activated, and photoreceptors are destroyed through the intrinsic apoptosis pathway. This mitochondrial scenario of AMD, may not only explain many of the previously puzzling features of AMD but offers an array of new therapeutic approaches for the disease targeted toward increasing mitochondrial energy production, decreasing mitochondrial ROS, and stabilizing the mtPTP.

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