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CKMT2 mutation in a patient with fatigue, age-related macular degeneration, deafness and atrial fibrillation

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Abstract

Purpose: The purpose of this study was to screen for mutations within mitochondrial creatine kinase (*CKMT*) genes, which encode for isoenzymes critical for high energy metabolism, such as that found in retina.

Methods: DNA was extracted from lymphocytes of clinically characterized age-related macular degeneration (AMD) patients (n=71). Flanking primers were used to polymerase chain reaction (PCR) amplify and sequence the exons, the open reading frame, and promoter regions of the *CKMT1A*, *CKMT1B* and *CKMT2* genes. An additional 72 individuals with AMD were screened for the novel *CKMT2* mutation in exon 4 (NM_001825.2 c.274C>T) by PCR and enzyme digestion. The RNA was extracted from cultured human retinal pigment epithelial cells (ARPE-19) and reverse transcript-PCR (RT-PCR) was performed with primers for the *CKMT2* gene.

Results: With our screening procedure, we identified 5 DNA variants in the *CKMT2* gene, 2 of which were novel (NM_001825.2 c.274C>T; 92Q>X, Exon 4 and NM_001825.2.-498G>GA; 5'UTR, Exon 1). The putative termination mutation in the *CKMT2* gene (NM_001825.2 c.274C>T; 92Q>X) was in a proband individual but lacking in his unaffected son and 142 unrelated AMD patients. Cultured human RPE cells can express the sarcomeric mitochondrial *CKMT2* gene product.

Conclusions: This is the first report of a termination mutation within the human *CKMT2* gene, which is critical for transfer of high-energy phosphate from mitochondria to creatine, a cytosolic carrier. Clinically, the subject had AMD, exercise fatigue, atrial fibrillation and deafness, all of which are known to be related to mitochondrial dysfunctions. Based upon our finding, we propose this *CKMT2* mutation may be a candidate gene for the phenotypes that include this quartet of symptoms.

Abbreviations: AMD: Age-Related Macular Degeneration; CKMT: Creatine Kinase; PCR: Polymerase Chain Reaction; ARPE-19: Human Retinal Pigment Epithelial Cell Line; RT-PCR: Reverse Transcript-PCR; mtDNA: Mitochondrial DNA; RPE: Retinal Pigment Epithelial; CK: Creatine Kinase; CT: Computer Tomography; *CKMT2*: Mitochondrial Creatine Kinase 2

Introduction

Organs such as brain, heart, muscle and retina have significantly higher energy demands as compared to other organs. Therefore, mutations or sequence variations in genes that contribute to energy generation through oxidative phosphorylation will have significant effects on the function of these organs.

Retinal tissues are extremely metabolically active and have numerous mitochondria in the inner segment of the photoreceptor cells. There is increasing evidence that alterations in the mitochondrial DNA (mtDNA) and in mitochondrial function may play a role in age-related macular degeneration (AMD), a retinal disorder that is a leading cause of vision loss in people over 60 years of age [1-8]. Atrophic AMD is a severe form of the disease that exhibits loss of the retinal pigment epithelial (RPE) cells and overlying photoreceptors. It has been reported that cultured RPE cells that are mitochondria deficient show an altered expression of creatine kinase (CK) genes [9]. These CK genes function to catalyze the transfer of high energy phosphates between ATP and creatine, thereby yielding ADP and phosphocreatine. *CKMT1A* and *CKMT1B* are ubiquitous mitochondrial genes that are on human chromosome 15q15.3, differ by only 2 nucleotides and code for an identical *CKMT1* protein. The *CKMT2* gene is located on human chromosome 5q13.3 and encodes for a sarcomeric mitochondrial creatine kinase isoenzyme. There are 3 transcript variants (NM_001825, NM_001099735 and NM_001099736) of the *CKMT2* gene, all of which encode the same 419 aa protein and have similar functions but are expressed in different tissues. All the *CKMT* genes are critical for energy generation.

In the present study, we initially screened the *CKMT1A*, *CKMT1B* and *CKMT2* genes from individuals with clinically significant AMD because these genes are highly conserved and critical for tissues

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with high energy requirements, such as the retina. Therefore, any variations in these genes could cause significant dysfunction and pathology for the effected retinas. With our screening procedure, we identified 5 DNA variants in the *CKMT2* gene, 2 of which were novel (NC.000005.10; hg19:chr5:81,252,816.274C>T; 92Q>X, exon 4 and hg19:chr5:81,233,356.498G>GA, exon 1). One proband subject had the putative termination mutation in the *CKMT2* gene (NM_001825.2) and thorough review of his medical status showed that in addition to AMD, he also had exercise fatigability, deafness and atrial fibrillation.

Materials and methods

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 performed according to the tenets of the Declaration of Helsinki for research involving human subjects.

AMD Classification

One hundred forty-three subjects underwent a complete dilated ophthalmic examination by Board certified ophthalmologists (D.S.B., A.B.N., and M.C.K.) including both slit lamp examination and an indirect ophthalmic exam with a 90 diopter lens or a fundus contact lens. Fundus photos, fluorescence and/or indocyanine green angiography were performed. The photos and angiograms were read by masked graders who were board certified retinal specialists. The subjects were graded according to the Clinical Age-Related Maculopathy Staging System (CARMS) [10]. 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. In this study the term Late 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 AMD patients were included in this study. Upon analyses, a putative termination heterozygous mutation NM_001825.2 c.274C>T (92Q>X) was found in one individual that we designated AMD-02-141 and further analyses of the proband subject and his son were conducted.

Clinical description of proband patient

Past ocular history: The proband subject is an 87 year old Caucasian male that has been treated for 6 years for the wet form of AMD. His ocular history is significant for cataract surgery for both eyes and YAG laser capsulotomy for his right eye in 1997. In January 2005, he developed a subretinal membrane in the left eye which was treated with photodynamic therapy and an intravitreal injection of Kenalog. In July 2006, his left eye received another treatment of photodynamic therapy combined with an Avastin intravitreal injection. Subsequently, he has developed dry AMD in the right eye and had best corrected visual acuity of 20/400 in both eyes. Dilated exam shows large macular glial scars with atrophy and age-related pigment alterations of the retinas (Figure 1).

Past medical history: The proband has loss of hearing (presbycusius), hyperlipidemia, chronic sinusitis, and benign prostatic hypertrophy. He also has experienced loss of exercise tolerance and becomes easily fatigued which caused him to give up the use of the rowing machine and his stationary bicycle training. The proband has a common form of atrial flutter of indeterminate duration and an EKG has shown atrial flutter with rapid ventricular response that inverted to atrial fibrillation with controlled ventricular response. In 1982, he was diagnosed with a history of paraoxysmal atrial flutter. A stress echo exam showed no ischemia, occasional premature ventricular complexes and moderately frequent premature atrial complexes. In 1999, his chronic sinusitis was evaluated and serial axial computer tomography (CT) scan of the paranasal sinuses showed marked mucosal disease in the left and to a lesser extent right anterior and posterior ethmoids. He had mucosal thickening in the maxillary sinuses (left>right), the periphery of the middle and inferior meati and the nasal septum along with an anterior nasal septal defect.

Past surgical history: His surgical history includes previous sinus surgery, vasectomy, extraction of kidney stones, appendectomy, bilateral osteomeatal surgery and herniorrhaphy.

Database accession numbers

Human reference sequences used for primer design and sequence analysis were accessed at NCBI and the accession numbers are as follows. *CKMT1a*: NM_001015001.1; *CKMT1b*: NM_020990.3; *CKMT2*: NM_001825.2, NM_001099735.1, and NM_001099736.1.

Extraction of total DNA

Venous blood samples (10 ml) from AMD patients were collected in tubes with 10mM ethylenediaminetetra-acetic acid (n=143). The Gentra Puregene DNA (Qiagen, Inc., Valencia, CA) extraction kit was used to isolate total DNA from leukocytes.

Sequencing

The sequencing primers used for the *CKMT1A*, *CKMT1B* and *CKMT2* are listed in Table 1. Primers were designed using PRIMER3 program (Whitehead Institute for Biomedical Research). All sequenced PCR products were treated to digest unused primers and nucleotides (ExoSAP-IT, USB Corp., Cleveland, OH). Sequencing was performed at the UCLA Sequencing and Genotyping Core facility. Sequence analyses were performed using the Mutation Surveyor

Table 1. Primer Sequences Used for Sequencing, PCR and RFLP.

Primer Sequence Forward Reverse		
CKMT2		
CKMT2E1	cggaggtctacgctgggattggtgagg	tcctccaaggcccttcacactggctct
CKMT2E2	caggcataagccaccatgcccagt	ctcatcccaagaacacagaggcccaca
CKMT2E3	tggctcctgcagttctcttgcccattg	tctcttgacctcgtgatctggccttcca
CKMT2E4	gggagcctagtggaaggatgggcaaaca	gccccatagagaagaaaaggaccccagga
CKMT2E5	acaaggggcaagtgaatggaaaggtct	gggcagtcacagtgctttccaggggta
CKMT2E6	tggcgattagaacccactgtggctgtg	aaggactgagcagagcaccaggagagg
CKMT2E7	tgggttggaacttgttcacctggcact	ccatttagctcagtctgggcatgctgtcaa
CKMT2E8	gcaatcaagctagggaggtaatgggaat	tggaagttaccatagtgtctttctttca
CKMT2E9	tctctggtgttagggatgtgctgaatg	tgggcagtgatgttttcttcctccaca
CKMT2E10	atgtcttttgtcagtaaacgcaccaat	tgtgaaaacggctaggctgtttgcttt
CKMT2E11	tggcaagttctcatttatcacagtgctgttcc	gaggtgtgtgtaagttgtggcaaaatca
CKMT1		
CKMT1AE1F	tctggattaaacagcattagggagaca	tgcctctgcttctcatgatcccccactt
CKMT1AE2F	ggagccccaccccctggagactgc	cccggattctcacttctacccgcacct
CKMT1AE3F	aaagccagacatggccaactggacagc	ggggctcagagtcttgggggaaagtga
CKMT1AE4F	gaaatcaatgcacaattgaagtct	aggaaataaggtgaaggggttggagat
CKMT1AE5F	aatgcctaaggaaggtctctcctttcc	tgggcctttcagagcccatgtctcatc
CKMT1AE6F	ggccagatgagacatgggctctgaaagg	ggcaaggcaagaattgggagagatagagg
CKMT1AE7F	cctcttttgtcttcatgccctcataaatgc	tggttttcccagtatggtcccagctggttt
CKMT1AE8F	tgtgggtctagctaagggagggtccag	aagggtccactcccctccccatcca
CKMT1AE9F	gctctgagcaggttcagggctctttcagg	catcccggaggctcactcactgccata
CKMT1AE10F	atgagcaggcaagtcagtcagtgataa	aatcccactgcctcttttcatttc
PCR		
β2-MG	ctcgcgctactctctctttctg	gcttacatgtctcgatcccactt
CKMT2-RNA	ggccagtatcttttctaagttgctaactgg	ttacttttgccaaactgaggcagagg

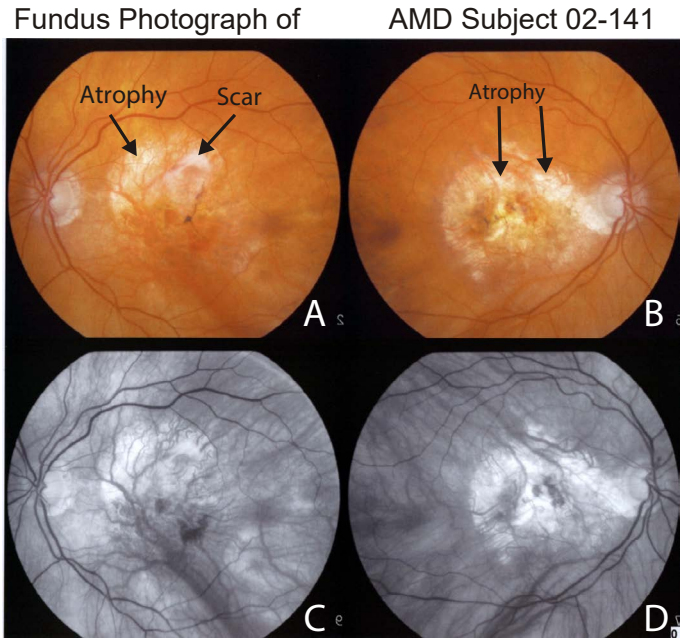

Figure 1. Fundus photography of the macular region of patient AMD-02-141 showing large area of macular atrophy, subretinal glial scars and pigmentary alterations of the retina (Panel A, left eye and Panel B, right eye). Red free photographs demonstrate regions with loss of retinal pigment epithelium throughout the macula of both eyes (Panel C, left eye and Panel D, right eye).

program, (Softgenetics, State College, PA). The DNA from 71 subjects underwent sequencing of all 11 exons of the *CKMT2* gene. In addition, the DNA from an additional 72 individuals with AMD were screened for the novel *CKMT2* mutation in exon 4 (NM_001825.2 c.274C>T) by PCR and enzyme digestion with Xba1 restriction enzyme (NEB, Ipswich, MA). The products were run on a 1.5% agarose gel stained with ethidium bromide.

Cell culture

ARPE-19 cells (ATCC, Manassa, VA) were grown in 1:1 mixture (vol/vol) of Dulbecco's modified Eagle's and Ham's nutrient mixture F-12; (Invitrogen-Gibco, Carlsbad, CA), nonessential amino acids 10mM 1×, 0.37% sodium bicarbonate, 0.058% L-glutamine, 10% fetal bovine serum, and antibiotics (penicillin G 100U/ml, streptomycin sulfate 0.1 mg/ml, gentamicin 10 µg/mL, amphotericin-B 2.5 µg/mL). Cells were plated into 60mm dishes and incubated at 37°C in 5% CO_2 until confluent.

RNA isolation

Human ARPE-19 cell cultures were rinsed in phosphate buffered saline (PBS, pH 7.2) and RNA was isolated using the RNeasy Extraction kit (Qiagen) following the manufacturer's protocol. Blood was obtained from patient AMD-02-141 and his son and the RNA was isolated using the Versagene Blood Kit (5 Prime, Gaithersburg, MD). One microliter of the RNA was analyzed for quantity using the NanoDrop (Thermo Scientific, Wilmington, DE).

cDNA synthesis and reverse-transcribed-PCR (RT-PCR)

All RNA samples were reverse transcribed into cDNA using the SMARTer kit (Clontech, Mountain View, CA). The cDNA from the ARPE-19 cells was amplified (Qiagen) with *CKMT2* primers (Table 1), annealing temperature of 67°C and PCR products were run on a 1% agarose gel and stained with ethidium bromide.

Results

A total of 20 AMD individuals were screened by sequencing for mutations in the *CKMT1A* and *CKMT1B* genes. Using flanking primers, all 10 exons for *CKMT1A* and *CKMT1B* were amplified and sequenced. Additionally, 500 bps of sequence upstream of the transcription start site of the *CKMT1A* and *CKMT1B* on human chromosome 15q15.3 were also screened for base variations. The coding regions for *CKMT1A* and *CKMT1B* were identical except for 4 base variations. During sequence analysis, these variations were used to distinguish the two genes. No new base variations were observed in the *CKMT1A* and *CKMT1B* genes (data not shown).

We then screened in 71 AMD patients all 11 exons of the sarcomeric *CKMT2* gene located on chromosome 5q13.3. In exon 4, we found a putative termination mutation heterozygous NM_001825.2 c.274C>CT (92Q>X) in one individual, patient AMD-02-141 (Figure 2). In addition, we also found 4 other DNA variations in the *CKMT2* gene in other AMD individuals. One of these 4 changes is a novel variation - 498G>GA (exon 1; Figure 3) found in patients AMD-02-30 and 02-44, while the others are known changes - 24191G>GA (rs2270823, exon 7), 33263 T>TC (rs34054011, exon 11), and 33440 G>GA (rs545, exon 11). The patient AMD-02-141 has an only son who was examined by a retinal specialist (DSB) and found to be phenotypically normal (age 55) with no signs of AMD. We then designed a restriction fragment length polymorphism (RFLP) assay as a means to rapidly and efficiently identify the truncating mutation c.274C>CT in the *CKMT2* gene. This assay was used to screen the proband and his son as well as 72 additional individuals with AMD. We determined that the son did not inherited the mutation c.274C>CT (92Q>X) (Figure 4) and we were not able to detect the 274C>CT mutation in any of the 72 additional AMD patients (Table 2).

Although there was no information on the expression of the different *CKMT2* transcripts in the retina, we wanted to determine if this gene was expressed within the human retinal RPE cells which are damaged early in AMD. The RNA was extracted from ARPE-19 cells and RT-PCR was performed. Figure 5 shows that the β2MG gene (334bp), representing nuclear DNA, was present in the ARPE-19 cells. Then RT-PCR was performed using primers for the *CKMT2* gene and showed a product of the desired size (1258bp), demonstrating that the *CKMT2* was expressed in human RPE cells. Interestingly, there was no *CKMT2* gene expression from lymphocytes samples isolated from subject 02-141 and his son (data not shown). This was not totally unexpected since *CKMT2* is usually associated with muscle cells and not blood cells.

In the *CKMT2* gene there are 19 non-synonymous SNPs that can cause amino acid changes as reported by NCBI Reference Assembly. Examination of the sequence patterns in the 71 AMD samples showed no mutations in these non-synonymous SNPs indicting that the *CKMT2* gene is highly conserved.

Discussion

Recently it has been shown that AMD retinas have significant mitochondrial abnormalities including mtDNA damage [1-5]. In addition, studies on the mtDNA ancestral variants show that Northern European mtDNA haplogroups (J, T, and U) are associated with increased incidence of AMD [7,8,11-17]. Mutations associated with mitochondria are often associated with disorders of multiple systems. Tissues that have high energy requirements are susceptible to damage if mitochondrial dysfunction occurs due to defects in the mtDNA (i.e.,

Table 2. CKMT2 SNP Results.

Exon	**1**	**4**	**7**	**11**	**11**	**11**	**4**
SNP	498 G>GA	274 C>CT	24191 G>GA	33263 T>TC	33440 G>GA	C>A	G>T
rs	Novel	Novel	rs2270823	rs34054011	rs545	rs15497	rs1063543
AMD: Sequencing							
	GG: 67/71	CC: 70/71	GG: 50/68	TT: 64/66	GG: 42/66	CC: 71/71	GG: 71/71
	GA: 04-71	CT: 1/71	GA: 15/68	TC: 2/66	GA: 18/66	CA: 0/71	GT: 0/71
	AA: 0/71	TT: 071	AA: 03/68	TT: 0/66	AA: 6/66	AA: 0/71	TT: 0/71
AMD: RFLP	N/A	CC: 72/72	N/A	N/A	N/A	N/A	N/A
		CT: 0/72					
		TT: 0/72					
Gene Coordinates hg19:chr5:NC_000005.10	81233356	81252816	81257052	81266124	81266301	81266178	81252762
Amino Acid Change	5' UTR	92 Q>X	Intron	Intron	3' UTR	394 L>M	74 A>S

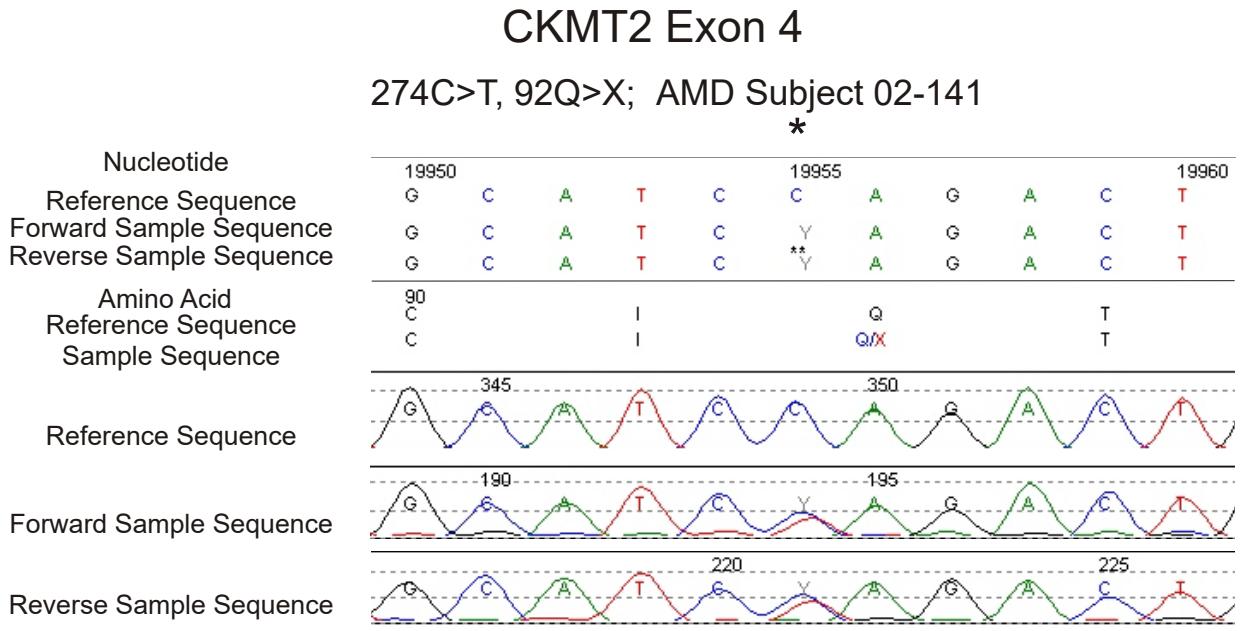

Figure 2. Sequencing of the *CKMT2* gene showing the 274C>T mutation at the hgy19:chr5:81,252,816 position in patient AMD-02-141.

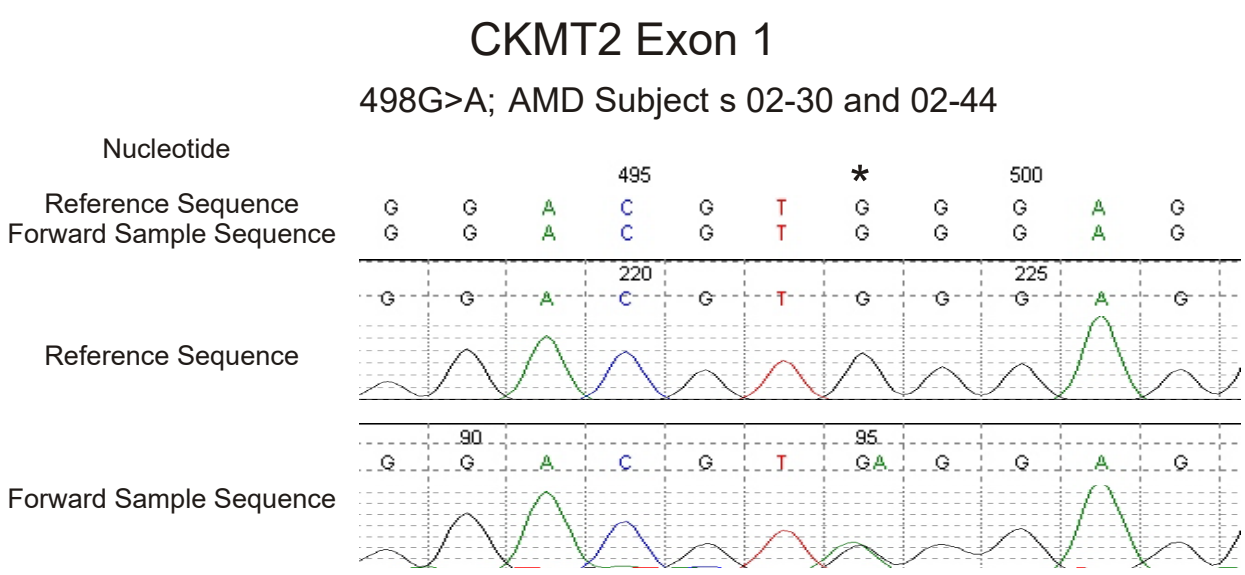

Figure 3. Sequencing of the *CKMT2* gene showing the novel 498G>GA variant at position hg19:chr5:81,233,356 in two AMD patients.

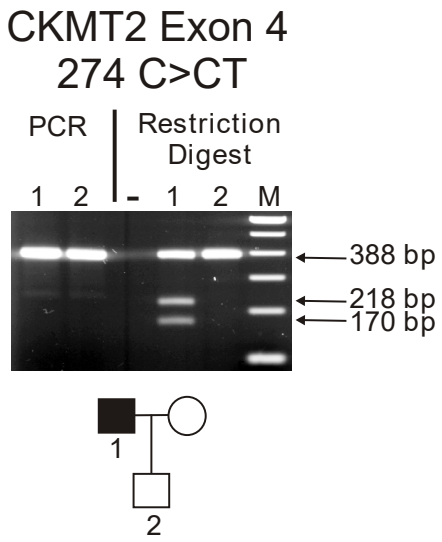

Figure 4. Upper panel: PCR amplification of the *CKMT2* in the exon 4 region from proband patient AMD-02-141 (lane 1) and his son (lane2) showed a 388 bp product. After enzyme digestion with Xba1 the proband-AMD-02-141 showed two lower bands (218bp and 170bp) indicating the presence of the 274 C>CT SNP while the son did not have the change (lane 2). Lower panel: Pedigree for proband AMD-02-141. M, marker.

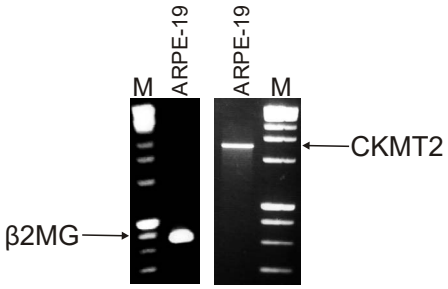

Figure 5. Gene expression of *CKMT2* was identified in human ARPE-19 cells. RT-PCR amplification of RNA isolated from human ARPE-19 cells showed expression of both the β2MG (334bp) and *CKMT2* (1258bp) genes. M, marker.

MELAS, Leigh Syndrome, Leber Hereditary Optic Neuropathy, Kerns-Sayre Syndrome or Chronic Progressive External Ophthalmoplegia) or within the nuclear genes that are associated with mitochondrial function. Common clinical features associated with mitochondrial disorders include exercise intolerance, cardiac dysrhythmias, pigmentary retinopathy, sensorineural deafness, axonal neuropathy and endocrine abnormalities [18]. Jones and coworkers reported the A3243G mtDNA mutation in a patient with early age related maculopathy, hearing loss, hypertension, ischemic heart disease, and asthma [19]. A clinical phenotype of macular pattern dystrophy, deafness, and diabetes has been reported in subjects with a mtDNA point mutation at A3243G, which is located within the mitochondrial tRNA(Leu) gene [20,21]. Individuals with the novel *MT-CYB* gene mutation (A15579G), affecting complex III activity, exhibit a phenotype involving severe exercise intolerance, deafness, mental retardation, retinitis pigmentosa, cataract, growth retardation and epilepsy [22]. Therefore, mutations within the mtDNA are associated with characteristic patterns of symptoms.

In the present study, we identified two novel SNP variants in the nuclear DNA *CKMT2* gene. One is a putative termination mutation (NM_001825.2 c.274C>T; 92Q>X, Exon 4) in an individual with AMD, exercise fatigue, atrial fibrillation, and deafness. Upon investigation, it is the first report of this mutation of the hg19:chr5:80548635 position in the *CKMT2* gene, indicating that it is a highly conserved gene throughout mammals. The second previously unreported SNP variant (NM_001825.2.-498G>GA; 5'UTR, Exon 1) was found in 4 out of 71 subjects. The *CKMT2* coordinates are at the 5q13.3 locus, located close to the defect causing Ushers Syndrome type 2C that includes deafness and retinitis pigmentosa [23].

Recent evidence demonstrates a relationship between mitochondria and the nuclear encoded *CKMT2* gene. Human retinal cells lacking mtDNA (*Rho0*) have significantly upregulated expression of the *CKMT2* gene [9]. While the *CKMT2* gene has not been linked to a recognized mitochondrial disease, it has been associated with increased severity and resistance to anti-thyroid drug treatments for endocrinology patients with T3-predominant Graves' thyroid disease [24]. *CKMT2* is a marker for myogenic differentiation that can be upregulated by a novel actin-binding protein called Striated Muscle Activator of Rho Signaling (STARS) [25,26]. Recent studies show that *CKMT2* and *CKMT1A/B* are exclusively produced in the highly metabolically active human brown fat but lacking in white adipose tissues [27]. The *CKMT*s utilize ATP to convert creatine (Cr) to phospho-creatine (p-Cr). The flow of metabolites is regulated by the mitochondrial creatine kinases, which structurally form complexes (mitochondrial interactosomes) with the mitochondrial voltage-dependent anion channel (VDAC) in the outer mitochondrial membrane and the ADP/ATP-translocase in the inner mitochondrial membrane. In this scenario, it becomes obvious that diminished levels or mutations of the *CKMT2* gene would have significant effects on energy homeostasis, production of reactive oxygen species and tissue longevity, likely leading to tissue dysfunction and diseases.

The earliest pathological changes in AMD are found in the RPE located beneath the neuro-retina. Our studies show *CKMT2* expression in cultured human RPE cells, which is in agreement with other studies [9]. However to our knowledge, this the first reported mutation within the *CKMT2* gene. The retina is among the most metabolically active tissues in the body and a mutation causing the production of a truncated form of *CKMT2* could result in diminished efficiency for cells, increased levels of apoptosis and RPE cell death, which are common features in AMD. Since mitochondrial creatine kinase is such an important protein for energy production, the *CKMT2* mutation would have serious consequences for all high energy requiring tissues and may potentially result in the multiple phenotypes described in our patient. Therefore, our data suggest that patients with the clinical picture of AMD plus deafness, exercise intolerance and/or cardiac dysarrhythmia should be screened not only for the mitochondrial DNA defect A3243G but also for the truncating mutation 274C>T in the *CKMT2* gene.

Acknowledgments

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Competing interests

The authors declare that they have no competing interests. This research was conducted in accordance with institutional review board approved procedures.

Author's contributions

MCK and NU helped design the study, interpret data, perform statistical analyses and write the manuscript. ABN and DSB performed clinical examinations on the patients and provided blood samples. NU designed the primers. SRA, DH, MM, and JL performed PCR and restriction enzyme digestion. SRA conducted sequencing analysis and figure generation. MC performed all tissue culture experiments.

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