Mitochondrial Sequence Changes in Keratoconus Patients

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Purpose. We investigated whether a group of patients with keratoconus (KTCN) harbor mutations in the mitochondrial genome.

Methods. We sequenced the full mitochondrial genome in a group of Saudi patients with KTCN ($n = 26$) and 100 ethnically matched controls who had no KTCN by examination.

Results. A total of 10 KTCN patients (38.5%) had potentially pathogenic nonsynonymous mtDNA mutations. Of the nonsynonymous sequence changes detected, 4 (40%) were in Complex I, one was in the $tRNA^{Glutamine}$, one was in $tRNA^{Tryptophan}$, one was in $tRNA^{Asparagine}$, one was in $tRNA^{Histidine}$, and two were in the $tRNA^{Leucine2}$. One nonsynonymous sequence change was heteroplasmic, whereas all the remaining 9 were homoplasmic. These sequence changes were not detected in controls of similar ethnicity. Four sequence changes were novel (were not reported previously) and 5 were reported previously. Additionally, we detected 54 synonymous (does not result in an amino acid change) sequence changes with no pathologic significance.

Conclusions. If our results are confirmed in a larger cohort and multiple ethnicities, then mtDNA mutation may be considered as a genetic risk factor contributing indirectly through the oxidative stress mechanism to the development and/or progression of KTCN.

Keywords: mitochondrial genome, keratoconus, mutation, Saudi Arabia

Keratoconus (KTCN) is a complex condition of multifactorial etiology. Genetic and environmental factors are associated with KTCN. Evidence of genetic etiology includes the condition's familial inheritance, discordance between dizygotic twins, and its association with other known genetic disorders. Several chromosomal loci and genes were reported to be associated with KTCN.[1,2] However, some eventually were excluded,[1,3] while in other studies, no confirmed association with the disease have been established.[4,5] This, of course, is not the case for the visual system homebox 1 (*VSX1*) gene, where mutations associated with KTCN cases have been found in different studies.[6–9] Having said that, there also are various studies, including our own study, that did not report *VSX1* mutations in cohorts of KTCN patients from various populations.[9–12] This indicates that KTCN is a complex condition of multifactorial etiology and that mutations in the *VSX1* gene cannot be responsible for all cases of KTCN. Genome-wide association study (GWAS) allows the interrogation of the whole genome in one experiment. A few candidate KTCN genes were identified in GWAS, including *IL1B*,[13] *CDH11, NUB1, COL27A1*, and *HGF*. A recent GWAS study suggested that SNP rs4954218, located near the *RAB3GAP1* gene, reported previously to be associated with corneal malformation, is a potential susceptibility locus for KTCN.[14] As these findings were relatively recent, it awaits conformations in larger cohorts and in multiethnicities.

It was reported previously that mitochondrial oxidative stress in Tet-mev-1 mice causes excessive apoptosis in several tissues leading to precocious age-dependent corneal physiologic changes, delayed corneal epithelialization, decreased corneal endothelial cells, thickened Descemet's membrane, and thinning of parenchyma with corneal pathologic dysfunctions, such as keratitis, Fuchs' corneal dystrophy (FCD), and probably KTCN.[15] Under transmission electron microscopy (TEM), swelling of the mitochondria were observed in KTCN corneal tissues.[16] The KTCN corneas exhibited more mtDNA damage than do normal corneas.[17] The KTCN fibroblasts had increased basal generation of reactive oxygen species and were more susceptible to stressful challenges (low pH and/or H_2O_2 conditions) than were normal fibroblasts.[18] Additionally, cultured KTCN fibroblasts have an inherent, hypersensitive response to oxidative stress that involves mitochondrial dysfunction and mtDNA damage. As a result, it was suggested that KTCN fibroblast hypersensitivity may have a role in the development and progression of KTCN.[19] To the best of our knowledge, no studies have investigated whether KTCN patients harbor mtDNA mutations. Here, we sequenced the full mitochondrial genome in a group of Saudi patients with KTCN.

Materials and Methods

Study Population

The study adheres to the tenets of the Declaration of Helsinki, and all participants signed an informed consent. The study was approved by the College of Medicine ethical committee (proposal number 09-659). All study subjects were self-identified as Saudi Arabian ethnicity. Family names all were present in the database of Arab families of Saudi Arabian origin. Additionally, these names indicated that all five major Saudi Arabian provinces were represented in the study population.

Table 1. Potentially Pathogenic Nonsynonymous mtDNA Sequence Changes Detected in KTCN Patients

Nucleotide Substitution	Codon	Location	% in Patients	% in Controls	Heteroplasmy	Novel	Interspecies Conservation
m.4218 T>A	Y304X	ND1	3.85	0	Yes	No	High
m.4381 A>G	–	t-RNA Gln	3.85	0	No	Yes	Moderate
m.5567 T>C	–	t-RNA Trp	3.85	0	No	No	High
m.5664 A>G	–	t-RNA Asn	3.85	0	No	Yes	High
m.11393 C>T	L212F	ND4	3.85	0	No	Yes	High
m.12178 C>T	–	t-RNA His	3.85	0	No	No	High
m.12308 A>G	–	t-RNA Leu2	3.85	0	No	No	High
m.12310 insA	–	t-RNA Leu2	3.85	0	No	No	High
m.12504 G>A	C56W	ND5	3.85	0	No	Yes	Moderate
m.14000 T>A	L555Q	ND5	3.85	0	No	No	High

Patients ($n = 26$), controls ($n = 100$).

Patients ($n = 26$) were selected from the anterior segment clinic at King Abdulaziz University Hospital after examination by two of the authors (AMA-M and HK). Patients were diagnosed with KTCN if the Schimpff-flow–based elevation map showed posterior corneal elevation within the central 5 to ≥+20 μm, inferior-superior dioptric asymmetry (I-S value) > 1.2 diopters (D), and the steepest keratometry > 47D. Patients were labeled as sporadic after examining the immediate family members and identifying the patient as isolated case of KTCN. Exclusion criteria were refusal to participate or post-LASIK ectasia.

The controls ($n = 100$) were recruited from the general ophthalmology clinic that had no ocular disease(s) or previous ophthalmic surgeries. Their slit-lamp exam showed clear cornea and their Schimpff-flow–based elevation map was within normal limit.

All KTCN cases secondary to causes, like trauma, surgery, Ehlers-Danlos syndrome, osteogenesis imperfecta, and pellucid marginal degeneration, were excluded from the study.

Sample Collection and DNA Extractions

Ficoll-Paque-PLUS (Pharmacia Biotech AB, Uppsala, Sweden) was used for lymphocyte separation and isolation from peripheral blood as detailed previously.[20] DNA was extracted from whole blood samples of all KTCN patients and controls using the PUREGENE DNA isolation kit from Gentra Systems (Minneapolis, MN).

DNA Amplification and Sequencing

The entire coding region of the mitochondrial genome was amplified in 24 separate PCRs using single set cycling conditions as detailed previously[21] for all patients and controls. Each successfully amplified fragment was sequenced directly using the BigDye Terminator V3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA), and samples were run on the ABI prism 3100 sequencer (Applied Biosystems).

Sequence Analysis of the Mitochondrial DNA Coding Region

Sequencing results were compared to the corrected Cambridge reference sequence.[22] All fragments were sequenced in forward and reverse directions at least twice for confirmation of a detected variant. Patient mtDNA sequences were compared to those from local controls, and all sequence variants were compared to the MITOMAP database,[22] the Human Mitochondrial Genome Database (available in the public domain at http://www.genpat.uu.se/mtDB), GenBank (available in the public domain at http://www.ncbi.nlm.nih.gov/Genbank/index.html), and Medline-listed publications.

Prediction of Pathogenicity

Pathogenic characteristics of a previously undescribed (novel) nonsynonymous mtDNA sequence changes were determined according to a combination of standard criteria,[23] an evaluation of interspecies conservation using the Polymorphism phenotyping v2 (PolyPhen-2 database, available in the public domain at http://genetics.bwh.harvard.edu/pph2/) and, when necessary, the Mamit-tRNA website (available in the public domain at http://mamit-trna.u-strasbg.fr/index.html), the human mitochondrial genome database (MitoMap website, available in the public domain at http://www.mitomap.org/MITOMAP), and available English literature. Therefore, a nonsynonymous nucleotide change was considered potentially pathogenic if it was not reported in mitochondrial databases or Medline-listed literature as a confirmed polymorphism, it was not present in local controls, it changed a moderately or highly conserved amino acid, it occurred in a region of high interspecies conservation, and it was assessed as possibly or probably pathogenic by PolyPhen-2.

For previously reported nonsynonymous nucleotide changes, consideration was given to pathogenic status determined by others and by mitochondrial databases in addition to the criteria described above.

Results

Table 1 displays the 10 nonsynonymous (resulting in an amino acid change) mtDNA sequence variants detected in 10 unique patients (each patient had one unique mutation) after sequencing the full mitochondrial genome in our KTCN patient group ($n = 26$). A total of 16 KTCN patients had no known or those we believe are potentially pathologic sequence changes. The 10 nonsynonymous variants listed in Table 1, are those that we believe were potentially pathologic based on the stringent criteria described in the Methods.

Of the nonsynonymous sequence changes listed in Table 1, 4 (40%) were in complex I, one was in the $tRNA^{Glutamine}$, one was in $tRNA^{Tryptophan}$, one was in $tRNA^{Asparagine}$, one was in $tRNA^{Histidine}$, and two were in the $tRNA^{Leucine2}$. One nonsynonymous sequence change was heteroplasmic, whereas all the remaining 9 were homoplasmic. These sequence changes were not detected in controls of similar ethnicity. Four sequence changes were novel (were not reported previously) and 5 were reported previously.

As for the synonymous (does not result in amino acid change) sequence changes, we detected 54. Of those, 50 (92.6%) were in complex I (NADH dehydrogenase, also called NADH:ubiquinone oxidoreductase), 3 (5.5%) were in complex IV (cytochrome c oxidase), and 1 (1.9%) in complex III (cytochrome bc1 complex, Table 2). All synonymous mtDNA

Table 2. Synonymous Sequence Changes Detected in KTCN Patients and Controls

Nucleotide Substitution	Codon	Location	% in Patients	% in Controls	Heteroplasmy	Novel
m.3357 G>A	M17M	ND1	3.85	4	No	No
m.3594 C>T	V96V	ND1	11.5	15	No	No
m.3603 C>T	N99N	ND1	3.85	4	No	Yes
m.3666 G>A	G120G	ND1	3.85	5	No	No
m.3768 A>G	L154L	ND1	3.85	5	No	No
m.3847 T>C	L181L	ND1	14.3	17	No	No
m.4059 C>T	S251S	ND1	3.85	6	No	No
m.4092 G>A	K262K	ND1	3.85	5	No	No
m.4104 A>G	L266L	ND1	11.5	15	No	No
m.4529 A>T	T20T	ND2	3.85	5	No	No
m.4769 A>G	M100M	ND2	11.5	16	No	No
m.4991 G>A	Q174Q	ND2	8.69	9	No	No
m.5237 G>A	P256P	ND2	3.85	4	No	No
m.9965 T>C	Y253Y	CO III	3.85	4	No	Yes
m.10115 T>C	I19I	ND3	11.5	15	No	No
m.10238 T>C	I60I	ND3	11.5	19	No	No
m.11009 T>C	L84L	ND4	3.85	8	No	No
m.11056 A>G	L99L	ND4	3.85	6	No	Yes
m.11251 A>G	L164L	ND4	8.7	9	No	No
m.11344 A>G	M195M	ND4	3.85	9	No	Yes
m.11437 T>C	A226A	ND4	11.53	17	No	No
m.11467 A>G	L236L	ND4	3.85	5	No	No
m.11485 T>C	G242G	ND4	3.85	6	No	No
m.11569 T>C	I270I	ND4	3.85	5	No	Yes
m.11620 A>G	A287A	ND4	3.85	5	No	No
m.11653 A>G	V298V	ND4	3.85	5	No	No
m.11719 G>A	G320G	ND4	61.53	71	No	No
m.11761 C>T	Y334Y	ND4	3.85	5	No	No
m.11944 T>C	L395L	ND4	11.53	13	No	No
m.11983 C>T	L408L	ND4	3.85	5	No	Yes
m.12372 G>A	L12L	ND5	8.69	9	No	No
m.12501 G>A	M55M	ND5	11.53	14	No	No
m.12612 A>G	V92V	ND5	8.69	10	No	No
m.12693 A>G	K119K	ND5	11.53	14	No	No
m.12696 T>C	Y120Y	ND5	3.85	4	No	No
m.12705 C>T	I123I	ND5	46.15	51	No	No
m.12816 C>T	A160A	ND5	3.85	4	No	No
m.12843 T>C	I169I	ND5	3.84	5	No	Yes
m.12879 T>C	G181G	ND5	3.84	5	No	No
m.13111 T>C	L259L	ND5	3.84	6	No	No
m.13174 T>C	L280L	ND5	3.84	6	No	No
m.13188 C>T	T284T	ND5	23.07	28	No	No
m.13422 A>G	L362L	ND5	3.84	6	No	No
m.13590 G>A	L418L	ND5	11.53	13	No	No
m.13650 C>T	P438P	ND5	8.69	10	No	No
m.13803 A>G	T389T	ND5	11.53	12	No	No
m.14070 A>G	S578S	ND5	3.84	5	No	No
m.14364 G>A	L104L	ND6	3.84	5	No	No
m.14470 T>C	G68G	ND6	8.69	10	No	No
m.14544 G>A	L44L	ND6	8.69	12	No	No
m.14566 A>G	G36G	ND6	11.53	12	No	No
m.14783 T>C	L13L	CYTB	8.69	10	No	No
m.15043 G>A	G99G	CYTB	11.42	13	No	No
m.15148 G>A	P134P	CYTB	3.84	7	No	No

sequence changes listed in Table 2 also were detected in controls with similar frequencies.

As for mitochondrial haplogrouping, we detected 12 different mtDNA haplogroups among our patient group (n = 26). We detected mtDNA haplogroup R0a in 3 patients, L3c in 3, N1a3 in 3, and J1b8 in 3, while 2 patients had the H63a, 2 had the L4A, 3 had the HV, 3 had the K1b1a, and one each had the mtDNA haplogroups U8, N2, M12G, and J1a.

Discussion

We enrolled 26 KTCN patients into this unique study, which was conducted to investigate whether KTCN patients possessed a pathogenic mtDNA sequence change. The 26 KTCN patients were found to lack mutations in the *VSX1* gene[12] and also lack any chromosomal abnormalities,[24] and, thus, the genetic causes, if any, for these KTCN cases still are not known.

Previous studies had indicated that oxidative stress, mitochondrial dysfunction, and mtDNA damage may have a role in the development and progression of KTCN.[15,17–19] Therefore, we investigated whether our KTCN patients possessed a pathogenic mtDNA mutation(s) in their mitochondrial genome. To our knowledge, this the first study that investigated an mtDNA mutation possible link to KTCN. We previously carried out similar studies in other ophthalmic diseases, such as Leber's hereditary optic neuropathy (LHON),[25] LHON-plus,[26] primary open angle glaucoma,[27] primary angle closure glaucoma,[28] and pseudoexofoliation glaucoma.[29]

Stringent criteria were used to assess the potential pathogenic status of the nonsynonymous mtDNA sequence changes detected here. Usually, the mitochondrial genome is highly mutated and one must be extremely careful in assigning pathogenic status to various sequence changes. In our cohort, only 38.5% (10/26) had mtDNA sequence changes that were potentially pathogenic. Most of the mutations detected were in complex I, a situation somehow resembling those for LHON. None of our KTCN patients had any of the three primary LHON mutations described previously,[30] although one mutation (m.4381 A>G) in the t-RNA^{Gln} was reported previously in LHON patients[25] who lack any of the three primary LHON mutations. This sequence change is located in pairing chain position 53 of the t-RNA^{Gln}, just before the TψC loop. PolyPhen could not predict the effect of this sequence change due to lack of data; however, phylogenetic data for 32 different species (Mamit-tRNA website; available in the public domain at http://mamit-trna.u-strasbg.fr/index.html) showed the sequence to be moderately conserved. This sequence alteration was not reported previously as a polymorphism and was absent in controls, and, therefore, was considered potentially pathogenic.

As for the m.4218 T>A, which is the only heteroplasmic mutation detected, it results in the introduction of a premature stop-codon in the ND1 gene. The high level of heteroplasmy (74%), the fact it results in premature stop-codon, and the fact that it was not detected in controls indicate that this sequence change is potentially pathogenic. As for m.5567 T>C, this mutation was reported previously in cases of mitochondrial encephalomyopathy[31] and was not found in our controls. Its location and its high interspecies conservation made us rank this as potentially pathogenic. As for the m.12308 A>G, this mutation was reported previously in the patients with chronic progressive external ophthalmoplegia (CPEO).[32] This mutation was not found in our controls and, similar to other tRNA mutations, was highly conserved, and, therefore, it is potentially pathogenic. The rest of the mutations in Table 1 (5664, 11393, 12178, 12310, 12504, and 14000) were not reported previously in association with diseases. We applied the same pathogenic criteria and we concluded that they are potentially pathogenic.

As for mtDNA haplogroups, we detected 12 different mtDNA haplogroups in our relatively small cohort. There was no particular mtDNA haplogroup that could confer susceptibility to KTCN, at least in our small cohort. We believe that a larger study will determine whether mtDNA haplogroups may confer susceptibility to KTCN or not.

Detecting potentially pathogenic mtDNA sequence changes in 38.5% (10/26) of our KTCN cohort certainly is a sign that mtDNA mutations may have a role in KTCN pathogenesis. We reported a fairly small group of patients from a restricted ethnic population, and this type of evaluation must be repeated in other centers. If these results are confirmed, then mtDNA mutation may be considered as a genetic risk factor contributing indirectly through the oxidative stress mechanism to the development and/or progression of KTCN.

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