



LACK OF MITOCHONDRIAL DNA DELETION(S) IN PATIENTS WITH KERATOCONUS

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Article Info <i>Received 15/11/2015</i> <i>Revised 27/11/2015</i> <i>Accepted 15/12/2015</i> Key words: Keratoconus, mitochondrial DNA	ABSTRACT Purpose to determine whether patients with Keratoconus and have evidence of mitochondrial DNA (mtDNA) deletions. Twenty seven Saudi Arabian patients with Keratoconus were recruited in this study. We compare their results to our database which contains mtDNA deletion information for 150 Saudi healthy controls free of systemic and ocular disease including Keratoconus. None of the Keratoconus patients screened had evidence of mtDNA deletion(s). mtDNA deletion(s) were not detected in any of the patients tested here. Other genetic or epigenetic factors cannot be excluded as a possible contributing factor to Keratoconus pathogenesis.
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INTRODUCTION

Keratoconus (KTCN; OMIM 148300) is a non-inflammatory thinning and anterior protrusion of the cornea that results in steepening and distortion of the cornea, altered refractive powers, and altered visual acuity. In more advanced cases, corneal scarring from corneal edema and decompensation further reduces visual acuity. Symptoms are highly variable and depend on the stage of progression of the disorder [1]. The incidence of Keratoconus ranges between 1/500 to 1/2000 individuals throughout the world [2]. The disease occurs with no ethnic or gender preponderance and causes significant visual impairment [2-4]. Most cases of Keratoconus are sporadic but a minority (5-10%) has positive family history [3,5]. In such cases both autosomal recessive and dominant patterns of inheritance have been reported [6-9]. There are several chromosomal loci and genes reported to be associated with Keratoconus [3,9]. However, some were eventually excluded [3,10], while others no confirmed association with the disease have been established [11,12]. This is not the case for the visual system homeobox 1 (*VSX1*) gene where mutations associated with Keratoconus cases have been found in different studies [13-16].

Other studies did not report *VSX1* mutations in diverse population cohorts of Keratoconus patients [17,18], including the Saudi Keratoconus patients [19]. This indicates that Keratoconus is a complex condition of multifactorial etiology and that mutations in the *VSX1* gene are not responsible for all cases of Keratoconus.

Keratoconus can be divided into three broad categories: i) Keratoconus associated with rare genetic disorders (such as Down syndrome, Nail-patella syndrome, Neurofibromatosis, etc); ii) Keratoconus in the setting of commonly reported associations (contact lens wear, eye rubbing, Atopy, Leber's congenital amaurosis, Mitral valve prolapsed and positive family history) and iii) Isolated Keratoconus with no associations. To our knowledge, no study has investigated chromosomal copy number variations in patients with any of the three types of Keratoconus mentioned above.

Here we investigate the possible presence of mitochondrial DNA (mtDNA) deletions in a group of Keratoconus patients from Saudi Arabia. To our knowledge, this investigation was not carried out anywhere else previously.



MATERIALS AND METHODS

Patients and Controls

Patients (n= 27) were selected from the anterior segment clinic at King Abdulaziz University Hospital (KAUH), King Saud University in Riyadh, Saudi Arabia after full ophthalmological examination by anterior segment specialists. Patients were diagnosed with Keratoconus if the Schimpff-flow based elevation map showed posterior corneal elevation within the central 5 mm $\geq +20 \mu\text{m}$, inferior-superior dioptric asymmetry (I-S value) >1.2 Diopter (D) and the steepest keratometry >47 D [20-22].

We have chosen these parameters to exclude cases that are Keratoconus suspects and to confine our study group to only cases with definite Keratoconus. All our patients were examined by a specialist and established to be free of any genetic disorder commonly associated with Keratoconus [3,21]. Patients were labeled as sporadic after examining the immediate family members and identifying the patient as isolated case of Keratoconus. Exclusion criteria was post-LASIK (laser-assisted in situ keratomileusis), ectasia or has a family history of Keratoconus or more than one individual from the same immediate family were affected. All study subjects were self identified of Saudi Arabian ethnicity. Family names were all present in the database of Arab families of Saudi Arabian origin. All Keratoconus cases secondary to causes like trauma, surgery, Ehlers Danlos syndrome, Osteogenesis Imperfecta and pellucid marginal degeneration were excluded from the study.

The controls were recruited from the general outpatients and ophthalmology screening clinic that had no systemic 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. This research adhered to the tenets of the Declaration of Helsinki, and all patients and controls signed an informed consent approved by the institutional review board.

Detection of mtDNA deletion(s)

The mtMinArc target spans the heavy strand origin of replication and is not affected by any of the reported large deletions. The mtMajArc target lies within

the “common” deletion as well as within 84% of the reported large deletions in the major arc. Primers and probes used are listed in Table-1. Real-time PCR of the three targets was performed using a probe-based multiplex qPCR assay on the 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) using the following thermal profile: 95°C for 10 min; 40 cycles of 95°C for 15 s, 55°C for 15 s; and 60°C for 1 min. The reaction components are as follows (given in final concentration): 22.5 μL TaqMan Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems), each mitochondrial primer at 50 nM (FmtMinArc, RmtMinArc, FmtMajArc, RmtMajArc), each nuclear DNA primer at 1250 nM (Fb2M, Rb2M), and each dual hybridization probe at 250 nM (PmtMinArc, PmtMajArc, Pb2M) (TaqMan MGB Probe, Applied Biosystems). The final reaction volume is 25 μL .

Absolute quantification was obtained using a standard curve generated from a reference DNA sample from a healthy, 30-year-old male. Whole blood was extracted using the QIAmp DNA Blood Mini Kit (Qiagen, Inc., Hilden, Germany) and the respective DNA concentrations were then measured independently using the following singleplex qPCR assays: mtDNA was quantified using a mtDNA qPCR assay³⁶ and nuclearDNA was quantified using Quantifiler DuoDNA Quantification Kit (Applied Biosystems). The sample was concentrated and requantified (using the same aforementioned singleplex qPCR assays) to generate the first standard, Std1, with a target concentration of approximately 7000 cells/mL and 2.7 million mtDNA copies/mL (exact quantification values used for absolute quantification). The deletion level was calculated by dividing the values for mito deletion / values for mito no deletion x 100.

RESULTS

None of our patients had a family history of Keratoconus and all were the only affected members of their respective family. The screening of the 27 Keratoconus patients revealed that none of our patients had mtDNA deletion in the mitochondrial genome known to be a hot spot for mtDNA deletions associated with various diseases. Therefore, our results are largely negative. The mtDNA deletion values ranged from 0.95 - 1.1 indicating the presence of no deletion.

Table 1. Primers and probes used

Primer name	Sequence
Mito-no deletion-Forward	CTA AAT AGC CCA CAC GTT CCC
Mito-no_deletion-Reverse	AGA GCT CCC GTG AGT GGT TA
Mito-deletion-Forward	CTG TTC CCC AAC CTT TTC CT
Mito-deletion-Reverse	CCA TGA TTG TGA GGG GTA GG
Mito-no_deletion_PROBE	FAM-CAT CAC GAT GGA TCA CAG GT
Mito-deletion_PROBE	NED-GAC CCC CTA ACA ACC CCC



DISCUSSION AND CONCLUSION

The 27 patients reported here met rigorous clinical criteria for clinically defined Keratoconus as detailed in methods. They were all sporadic cases and were identified as Saudi Arabians. We also recruited 150 healthy controls (free of Keratoconus). Mutations in *VSX1* gene have been identified in association with Keratoconus [13-15], but in our cohort, we did not detect any mutations in this gene [19]. This may not be surprising as the role of *VSX1* in Keratoconus is still ambiguous and many studies in various populations found no mutations in this gene in their respective populations [12,17,18,23]. Human *VSX1* is a member of the CVC domain containing paired-like class of homeo-proteins. *VSX1* expression in humans is detected in embryonic craniofacial, adult retinal, and adult corneal tissues. Previous studies have shown that the pathogenesis of Keratoconus is very complex and several gene- and gene-environmental interactions play a critical role in disease prognosis. Additionally, we have previously shown that Keratoconus patients do not possess a chromosomal aberration [deletion(s) / duplication(s)] [24]. However, we did find some potentially pathogenic mtDNA mutations in Keratoconus patients from Saudi Arabia [25]. In this study, we could not detect any mtDNA pathogenic deletions after screening our Keratoconus cohort. This is intriguing as usually mtDNA deletions presence are usually associated

with increase in mtDNA copy number. This is a form of compensation to oxidative stress where mtDNA replicates faster in order to generate more mitochondrion. The results this relatively small pilot study will pave the way to investigate the association of mtDNA aberration with Keratoconus and will certainly shed more light on the association of mtDNA copy number and mtDNA deletions.

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CONFLICT OF INTEREST:

The authors declare that they have no conflict of interest.

STATEMENT OF HUMAN AND ANIMAL RIGHTS

All procedures performed in human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This article does not contain any studies with animals performed by any of the authors.

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