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Role of extracellular matrix remodelling gene SNPs in keratoconus

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ABSTRACT

Introduction: Single nucleotide polymorphisms (SNPs) in genes for certain structural components may be implicated in the pathogenesis of keratoconus. We hypothesized links between SNPs in genes coding for collagen, matrix metalloproteinase 9 (MMP9) and tissue inhibitor of matrix metalloproteinase (TIMP) and keratoconus. Furthermore, we hypothesized links between MMP-9 and TIMP-1 SNPs and their tear level in keratoconus patients.

Materials and methods: We genotyped 200 keratoconus and 100 control subjects by allele-specific PCR, and quantified MMP-9 and TIMP1 in tear samples by ELISA.

Results: *COL4A3* (rs55703767) and *MMP-9* (rs17576) G alleles were over-represented in keratoconus patients ($P < 0.01$). *TIMP-1* (rs6609533) A allele was more prevalent in keratoconus females ($P < 0.01$) but not in males ($P = 0.73$). MMP-9 was higher ($P < 0.001$) and TIMP1 lower ($P < 0.001$) in tear samples from keratoconus patients compared to controls. Keratoconus cases carrying *MMP-9* (rs17576) homozygous (GG) alleles had higher tear MMP-9 compared to those carrying the (A) allele ($P < 0.01$). Females carrying *TIMP-1* (rs6609533) homozygous (AA) alleles in both groups had significantly lower tear *TIMP-1* compared to carriers of the AG and GG genotypes.

Conclusions: This study supports the hypothesis of a functional role for *COL4A3* (rs55703767, G/T), *MMP-9* (rs17576, A/G) and *TIMP-1* (rs6609533, A/G) SNPs in the pathogenesis of keratoconus.

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Introduction

Keratoconus is a corneal condition that commonly begins during the teenage years with ongoing corneal thinning which results in corneal protrusion, irregular astigmatism and decreased vision [1], and eventually transplantation is recommended to restore vision [2]. The cornea consists of an extracellular matrix, primarily different types of collagen [3]. In corneal disease, highly organized corneal architecture is disrupted, the extracellular matrix is degraded and a new disorganized matrix is formed [4]. Results of various studies suggest that corneal thinning, typical for keratoconus, may be linked to a decreased amount of total collagen, the main corneal protein and an alteration in extracellular matrix structure [5,6]. Other connective tissue disorders are also prevalent in keratoconus, suggesting an underlying structural abnormality that includes Ehlers Danlos syndrome [7] and mitral valve prolapse [1].

Several genes are implicated in pathogenesis of this condition [1,8–11], and those encoding collagen may be considered candidate genes [6,12,13]. Although there are several links between single nucleotide polymorphisms (SNPs) in collagen genes and keratoconus, most abnormalities in corneal collagen structure are unrelated to variation in collagen genes and, therefore, other different genetic factors are likely to be involved in the degradation of extracellular matrix components. Matrix metalloproteinases (MMPs) are extracellular endopeptidases that have a key role in extracellular matrix remodelling; they control intercellular interactions and interactions of cells with extracellular matrix, and cause degradation of various types of collagen and are upregulated during matrix remodelling [14–16]. Matrix metalloproteinase-9 (MMP9) and gelatinase are the chief matrix-degrading enzymes produced by human corneal epithelium [17].

Tissue inhibitors of matrix metalloproteinase (TIMPs) are specific inhibitors of MMPs which regulate the activity of MMPs in various tissues, and the balance between TIMPs and MMPs regulates extracellular matrix remodelling [18–22]. TIMP-1 presents a unique binding interaction with MMP-9 with high affinity and is normally secreted as a TIMP-1/MMP-9 complex [23]. *COL4A5* codes for collagen type 4. Corneal thinning, a clinical hallmark of keratoconus, is associated with the destruction of extracellular matrix by increased activity of proteolytic enzymes, including metalloproteinases, and decreased level of their inhibitors [24].

We hypothesized roles for potential candidate genes and gene variations in the pathogenesis of keratoconus, these being *COL4A3* (rs55703767, G/T), *MMP-9* (rs17576,

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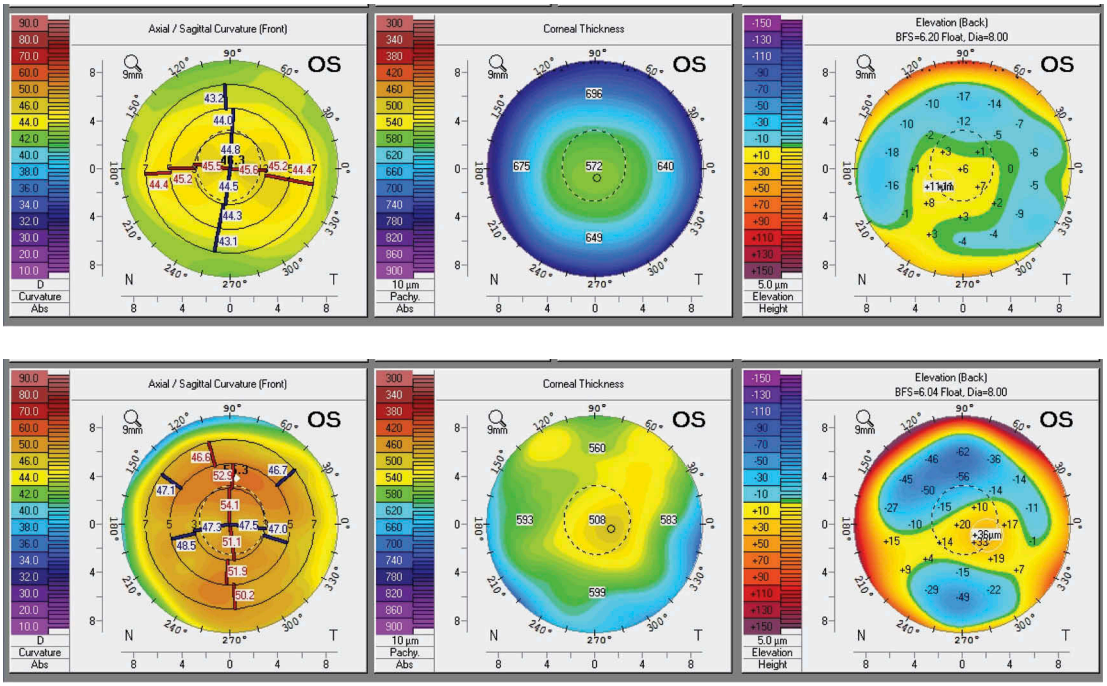

Figure 1. Elevation-based topography with pentacam representing the refractive 3 map display from left to right: anterior (front) elevation, pachymetry maps (corneal thickness) and posterior (back) elevation. The pentacam displays them in a colour-coded fashion; green, yellow and light blue for near normal values and red and purple for caution. A: normal eye, B: keratoconus.

A/G) and *TIMP-1* (rs6609533, A/G) which, being found at Xp11.3, call for analysis in each sex separately.

Patients and methods

We recruited 200 keratoconus patients seeking refractive surgery (84 men and 116 women; mean [SD] age 34.8 [5.8] years) and 100 age- and sex-matched unrelated control subjects (40 men and 60 women; aged 33.4 [6.04])($p = 0.7$ and $p = 0.68$, respectively). None of the participants had a history of previous refractive surgery. The study was approved by the Ethical Committee of Faculty of Medicine, Benha University, and informed consent was obtained from all participants. All subjects went through a complete ophthalmic examination which included refraction, visual acuity measurement, slit lamp biomicroscopy, retinoscopy, fundus examination in addition to conventional corneal topography and elevation-based topography with pentacam. Keratoconus was diagnosed if there was a scissoring reflex on retinoscopy and central or paracentral steepening of the cornea on topography with at least 1 of the following slit lamp findings: stromal thinning, anterior bulging of cornea, vogt striae, Fleischer ring, descemet's breaks, apical scars & subepithelial fibrosis [25]. The grade of keratoconus and the progression of steepening of the anterior surface of the cornea were also evaluated by pentacam (Figure 1) [26] and cases of all grades were involved in this study.

A plastic capillary tube was used for tear collection as previously described [27]. To take out tear fluid, the lower lid was pulled down and tear fluid was sucked from the conjunctival sac into a capillary tube, then pipetted out into an eppendorf tube. Collected samples were stored immediately at −80°C until analysis. DNA Preparation and Genotyping was as follows: A peripheral venous blood sample (about 3 mL) was collected into EDTA. Each sample was mixed and divided into two eppendorf tubes then stored at −80°C for further processing. QIAamp DNA blood mini kit (Qiagen, Germany) was utilized for DNA extraction following the manufacturer's directions. Extracted DNA concentration was assessed by NanoDrop 2000c Spectrophotometer (Thermo Scientific, USA). Readings were assessed at wavelengths of 260 and 280 nm [28]. Genotyping of the *COL4A3* rs55703767, *MMP-9* rs17576 and *TIMP-1* rs6609533 SNPs were performed using Allele-specific PCR.

For *COL4A3* (rs55703767, G/T) SNP detection, a common forward primer, 5'-CTGCATTTGGGAATCA TAGT- 3', was used. The reverse primer for the G allele was 5'-AGGATTACCTTAATGCCACC-3', and the reverse primer for the T allele was 5'-AGGATTACCTTAATG CCACA-3'. For *MMP-9* (rs17576, A/G) SNP detection, a common reverse primer, 5'GTGGAAAGACAAA CTGATGG-3', was used, with a forward primer for the G allele of 5'-CCCAGGACTCTACACCAG-3', and a forward primer for the A allele was 5'-CCCAGGACTCTACACCAA-3'. For the *TIMP-1* (rs6609533, A/G) SNP a common reverse primer, 5'-GGCTTCAAGATAGTCACTGG-3', was used, the forward primer for the G allele was 5'-CTGTGTCCAATACCGTGTGATAG-3', and the forward primer for the A allele was 5'-CTGTGTCCAATACCG

TGTGATAA-3'. DreamTaq Green PCR Master Mix (Thermo Scientific, Germany) was used for the genotyping reaction mixture of the three SNPs according to the manufacturer's instructions. PCR runs were performed using Biometra TAdvanced thermal cycler (Biometra, GmbH, Germany). The reaction mixtures were covered with mineral oil and subjected to the following thermal cycling parameters: 1 cycle of 95°C for 3 min (Initial denaturation) then 35 cycles of 95°C for 30 s (denaturation), 47–52°C for 30 s (annealing), 72°C for 30 s (extension) and 1 cycle of 72°C for 7 min (final extension).

Products of the PCR reactions were detected by electrophoresis using 2% agarose gel stained with ethidium bromide. The bands were visualized using UV Transilluminator (254 nm, Alpha Innotech Corporation, USA), then photographed and analysed using a digital camera (Olympus, ED lens, 6.3 megapixel, USA). Photos were transferred to computerized analysis through the Gel Documentation System (Alpha Innotech, USA). The PCR product size for the present alleles was 216, 275 and 306 bp for *COL4A3* (rs55703767, G/T), *MMP-9* (rs17576, A/G) and *TIMP-1* (rs6609533, A/G), respectively. ELISA kits for MMP-9 and TIMP1 were obtained from Abcam, Cambridge, UK. MMP-9 and TIMP1 quantification in tear fluid were determined according to the manufacturer's instructions.

Data were analysed using SPSS v24; results are displayed as median and interquartile range (IQR), numbers and percentages. In the statistical comparison of quantitative data between the different groups, the significance of difference was tested using Student's t-test and Mann-Whitney U-test. For categorical data between the different groups, chi-square test was used. Odds ratios (OR) with 95% confidence intervals (CI) were calculated. Significance was accepted at $p < 0.05$.

Results

Tear fluid MMP-9 was higher in keratoconus (58 (50–66) pg/mL) than in healthy controls (32 (25.5–39) pg/mL)($p < 0.001$). TIMP1 was lower in keratoconus (104 (69.5–136.5) pg/mL) compared to controls (169 (135–184) pg/mL) ($p < 0.001$).

The allelic frequencies and genotypic distributions of the *COL4A3* (rs55703767), *MMP-9* (rs17576) and *TIMP-1* (rs6609533) polymorphisms were compared between the keratoconus patients and healthy controls (Table 1). Regarding the genotypic distribution of the *COL4A3* (rs55703767, G/T) SNP, the GG genotype and the G allele were significantly over-represented in keratoconus and the T allele was significantly more prevalent in the control group. These findings indicated to the presence of an association between the *COL4A3* rs55703767 SNP and keratoconus. For the genotypic distribution of the *MMP-9* (rs17576) SNP, the GG genotype and the G allele were significantly over-represented in keratoconus. The AA genotype and the A allele were significantly more prevalent in the control group (Table 1).

The MMP-9 (rs17576) SNP could affect MMP-9 tear level in keratoconus patients, the tear levels of MMP-9 were significantly higher in individuals carrying the homozygous (GG) allele [61 (55–68) pg/mL] as compared to those carrying (A) allele (GA & AA) [47 (45–50)] ($p = 0.00168$). Regarding tear levels of MMP-

Table 1. Genotype distribution and allele frequencies of the COL4A3 (rs55703767), MMP-9 (rs17576) and TIMP-1 (rs6609533) polymorphisms in keratoconus patients and healthy controls.

SNP	Keratoconus patients, n (%)	Controls, n (%)	OR (95% CI)	*P* value
COL4A3 (rs55703767)				
GG	159/200 (79.5%)	68/100 (68%)	1.8 (1.06–3.14)	0.0298
GT	34/200 (17%)	24 (24%)	0.65 (0.36–1.17)	0.1496
TT	7/200 (3.5%)	8 (8%)	0.42 (0.15–1.19)	0.1008
Allele frequency				
G	352/400 (88%)	160/200 (80%)	1.83 (1.16–2.90)	0.0097
T	48/400 (12%)	40/200 (20%)	0.55 (0.35–0.86)	
MMP-9 (rs17576)				
GG	164/200 (82%)	72/100 (72%)	1.77(1.001–3.12)	0.048
AG	25/200 (12.5%)	12/100 (12%)	1.05(0.50–2.18)	0.9012
AA	11/200 (5.5%)	16/100 (16%)	0.31(0.14–0.69)	0.0041
Allele frequency				
G	353/400 (88.3%)	156/200 (78%)	2.12(1.35–3.33)	0.0011
A	47/400 (11.8%)	44/200 (22%)	0.47(0.30–0.74)	
TIMP-1(rs6609533) in females				
GG	26/116 (22.4%)	24/60 (40%)	0.43(0.22–0.85)	0.015
AG	59/116 (50.9%)	28/60 (46.7%)	1.18(0.63–2.28)	0.598
AA	31/116 (26.7%)	8/60 (13.3%)	2.37(1.01–5.55)	0.047
Allele frequency				
G	111/232 (47.9%)	76/120 (63.3%)	0.53 (0.34–0.83)	0.0061
A	121/232 (52.1%)	44/120 (36.7%)	1.88 (1.19–2.96)	
TIMP-1(rs6609533) in males				
GY	48/84 (57.1%)	24/40 (60%)	0.89(0.41–1.91)	0.7632
AY	36/84 (42.9%)	16/40 (40%)	1.13(0.52–2.42)	
Allele frequency in all subjects				
G	159/316 (50.3%)	100/160(62.5%)	0.61(0.41–0.89)	0.0120
A	157/316 (49.7%)	60/160(37.5%)	1.64(1.11–2.42)	

OR: odds ratio; CI: confidence intervals, significant.

9 in the control group, there was no statistically significant difference between individuals carrying the homozygous (GG) allele [31 (25–39) pg/mL] and those carrying (A) allele (GA & AA) [26 (25–33) pg/mL] ($p = 0.43$).

Regarding the genotypic distribution of the *TIMP-1* (rs6609533) SNP, results for males and females were investigated separately because TIMP-1 gene is located on the X chromosome. The genotypic distribution of the *TIMP-1* (rs6609533, A/G) SNP in females showed that the GG genotype and the G allele frequency were significantly over represented in the controls. The AA genotype and the A allele frequency were more prevalent in keratoconus patients. Keratoconus females carrying the homozygous (A) allele had significantly lower tear levels of TIMP-1 [72 (48–101) pg/mL] compared to carriers of the AG and GG genotypes [115 (83–147)] ($p = 0.023$). Also, females of the control group carrying the homozygous (A) allele had significantly lower tear levels of TIMP-1 [131 (124–137) pg/mL] as compared to carriers of the AG and GG genotypes [178 (161–190) pg/mL] ($p = 0.008$). In males, no statistically significant difference was detected in the genotype distribution between both groups. Analysing results of allele frequency in all subjects (males and females) revealed also that the G allele frequency was over-represented in the controls and the A allele frequency was more prevalent in keratoconus patients.

Discussion

Identification of functional polymorphisms in genes involved in keratoconus development and progression may help to understand of the molecular mechanisms of the pathogenesis of keratoconus. In this study, we aimed to evaluate the potential association of *COL4A3* (rs55703767, G/T), *MMP-9* (rs17576, A/G) and *TIMP-1* (rs6609533, A/G) SNPs in keratoconus, and investigated the association between *MMP-9* (rs17576, A/G) and *TIMP-1* (rs6609533, A/G) SNPs and their tear levels in order to assess the functional role of these SNPs.

Our findings showed significant differences between keratoconus patients and the control group regarding *COL4A3* rs55703767 G/T genotype distribution & allele frequencies as the GG genotype and the G allele were significantly over-represented in keratoconus patients whilst the T allele was significantly more prevalent in the control group. These findings corroborate the study of Stabuc et al. who reported that *COL4A3* D326Y (rs55703767) had significant differences in genotype distribution between keratoconus patients and the control group and that 976G (D326Y, *COL4A3*) is one of the significantly prevalent alleles in keratoconus [6]. However, Saravani et al. did not show significant representation of the GG genotype and the G allele in keratoconus patients but reported that the TT genotype as well as the T allele decreased the risk of keratoconus in Iranians [29]. Kokolakis et al. also found no association between *COL4A3* rs55703767 SNP and keratoconus risk in Greeks [30]. These differences may be due to the variable genotype distributions among different ethnic groups.

Regarding the *MMP-9* (rs17576, A/G) SNP, we demonstrate that the G allele is significantly over-represented in keratoconus, suggesting that it could be a risk factor. Saravani et al. did not show significant prevalence of the G allele in the Iranian keratoconus patients but reported that the A allele was significantly over-represented in the healthy controls [29]. These results were agreed with that of Hall et al. who reported the same findings in myopia patients; they demonstrated that the risk of myopia was highest in those homozygous for the G allele in exon 6 of the *MMP-9* R279Q (rs17576) [31]. Experimental and clinical evidence indicates that excessive ocular elongation associated with myopia is the result of altered extracellular matrix remodelling of the scleral shell controlled by changes in gene expression of matrix metalloproteinase enzymes that degrade matrix proteins and modulate sclera extensibility [31,32], this being the same mechanism hypothesized for keratoconus.

We also show that the MMP-9 tear level in keratoconus is significantly higher in individuals carrying the homozygous (G) allele as compared to those carrying (A) allele. The prevalence of the G allele in keratoconus and the higher MMP-9 tear level in individuals carrying the homozygous (G) allele in this study corroborates previous studies reported that the 279glutamine (G) allele in the coding region of the MMP-9 gene is in strong linkage disequilibrium with the _1562T allele in the promoter region of the same gene [33] and that the _1562T promoter and 279Q coding alleles are associated with higher plasma levels of the MMP-9 enzyme [34].

The *TIMP-1* (rs6609533, A/G) SNP results for males and females were analysed separately because TIMP-1 is a sex-linked gene located on X chromosome. In females, the GG genotype and the G allele frequency were significantly over-represented in controls. The AA genotype and the A allele frequency were significantly associated with keratoconus patients suggesting that the A allele could be a risk factor for susceptibility to keratoconus. Saravani et al. also reported that in females, AA increased the risk of keratoconus [29]. There have been few studies on the genetic polymorphisms of X-linked *TIMP-1* and their association with disease conditions, one of these is that of Kumar et al. on chronic obstructive pulmonary disease (COPD) patients where Protease-antiprotease imbalance has a great impact on COPD pathogenesis [35]. They demonstrated that the AA genotype was significantly higher in female patients as compared to controls and that the minor allele A of

intronic SNP rs6609533 of *TIMP-1* could be one of the risk factors impacted in COPD pathogenesis.

We also found that the tear level of TIMP-1 in female keratoconus patients was significantly lower in those carrying the homozygous (A) allele as compared to carriers of the AG and GG genotypes. In males, there were no statistically significant differences in the genotype distribution between both groups. These results are consistent with those of Saravani et al. who reported that the alleles were not associated with keratoconus risk/protection [29].

This work represents an advance in biomedical science because it demonstrates significant differences between keratoconus patients and the control group regarding *COL4A3* (rs55703767, G/T), *MMP-9* (rs17576, A/G) & *TIMP-1* (rs6609533, A/G) genotype distribution and allele frequencies, and demonstrates a functional role for *MMP-9* (rs17576, A/G) & *TIMP-1* (rs6609533, A/G) SNPs represented by their effect on MMP-9 and TIMP-1 tear levels. Levels of these molecules in tear sample may help future diagnosis and management.

Summary table

What is known about this subject:

- Keratoconus is a common corneal dystrophy with stromal thinning, protrusion, astigmatism and decreased vision.
- Corneal thinning may be underlined by a decreased amount of total collagen, the main corneal protein and an alteration in extracellular matrix structure.
- Collagen, MMP9 and TIMP genes may impact in keratoconus pathogenesis.

What this paper adds:

- This study identifies the impact of *COL4A3* (rs55703767), *MMP-9* (rs17576) and *TIMP-1* (rs6609533) SNPs on gene function and keratoconus risk.
- *COL4A3* (rs55703767) G allele, *MMP*-9 (rs17576) G allele and *TIMP-1* (rs6609533) A allele were significantly prevalent in keratoconus patients.
- MMP-9 was detected at significantly higher concentrations in tear fluid samples from keratoconus patients while TIMP1 was detected at significantly lower concentrations in tear fluid samples from keratoconus patients.

Disclosure statement

No potential conflict of interest was reported by the authors.

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