


Role of HGF polymorphisms in the development of keratoconus in South Asian population

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ABSTRACT

Background: Hepatocyte growth factor (HGF) has been previously reported to be causative of keratoconus (KC) in different populations.

Aims: The current study was conducted to investigate the role of HGF single nucleotide polymorphisms (SNPs) in sporadic KC cases from South Asian population of Pakistani origin.

Methodology: A total of 100 sporadic cases were screened KC associated reported polymorphisms in HGF (rs17501108 and rs3735520) using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method.

Results: No significant association of the HGF SNPs were observed in the sporadic cases of KC and possibility of involvement of another gene in the pathogenesis of KC exist.

Conclusion: Present study came with the findings that although globally an association exist between HGF and KC but genotyping data of Asian population of Pakistan origin does not show any association between HGF and pathogenesis of KC.

Keywords: keratoconus, sporadic keratoconus, hepatocyte growth factor

INTRODUCTION

Keratoconus (KC) is a corneal dystrophy characterized by bilateral cone shaped cornea due to the progressive thinning of the corneal stromal layers [1]. It is a progressive, non-inflammatory and multifactorial condition, where the corneal stromal thinning leads to severe refractive error, irregular astigmatism, and high myopia in the patients [2, 3], thus resulting in loss of visual acuity. In the early stages of the disease no physical symptoms are apparent, only a slight loss of visual acuity is experienced by the patients [4], as the disease progresses from moderate to severe stages, corneal protrusion, fleischer's ring, vogg's striae, and munson sign start appearing [4]. The estimated rate of incidence is one in 2,000 individuals but the disease prevalence varies worldwide, in Caucasians it is estimated to be 8.8-54.5 per 100,000 individuals [5], while in Malaysians the estimate is one per 100 person [6]. It was reported the incidence of KC in Iran (760:100,000) higher than the Western world [7].

The disease manifests itself mostly in the second decade of life, which progresses until the third and fourth decade [8], in certain populations males are more prone to develop the disease than females [9, 10]. Sporadic occurrence of KC is more common than familial cases; in addition, individuals with a positive family history are at greater risk of suffering from the disease. In familial cases autosomal dominant mode of inheritance is more frequent than X-linked or recessive

inheritance [11, 12]. The exact etiology of KC is still unknown, however, being a multifactorial disease, it is expected that environment and genetics probably play a combined role in disease pathogenesis [13].

Genome-wide association studies (GWAS) and linkage analysis of KC patients of different ethnicities have indicated various single nucleotide polymorphisms (SNPs) and loci to be associated with KC [14], including lysyl oxidase (LOX), tissue inhibitor of metalloproteinases-3 (TIMP3), collagen type VI alpha-1 chain (COL6A1), matrix metalloproteinase 9 (MMP9), visual system homeobox 1 (VSX1), hepatocyte growth factor (HGF), superoxide dismutase 1 (SOD1), zinc finger e-box binding homeobox 1 (ZEB1), and crumbs homolog 1 (CRB1).

Among all these genes, HGF encoded protein is a paracrine mediator of stromal-epithelial interactions and plays a significant role in corneal epithelial wound healing, cell proliferation, motility, and differentiation, all of which are involved in maintaining corneal homeostasis and wound repair mechanism [15].

The genetics of KC has not been investigated yet in South Asian population, therefore the present study was conducted to determine the role of HGF in south Asian KC patients of Pakistani origin by performing case-control association study for the sporadic KC cases.

Table 1. Primers for the amplification of HGF polymorphisms

Gene	SNPs	Primer sequence	Annealing (°C)	Product size (bp)	MgCl ₂ (mM)	Primer (μM)
HGF	rs17501108	F: 5'-TTAAGCCCAGGTTTCTCCAC-3'	61	216	3.0	0.4
		R: 5'-CAGAAACAGAATGGCACCTG-3'				
HGF	rs3735520	F: 5'-AAATGGATCCTGGGGACAC-3'	61	242	2.5	0.6
		R: 5'-TCCCTCCACATACCACCTTC-3'				

Note. HGF: Hepatocyte growth factor; F: Forward primer; & R: Reverse prime

METHODS

Sample Collection and DNA Isolation

A total of 100 sporadic KC cases were collected from local hospitals. Sampling of the patients was done regardless of age, sex (males=64, females=36) and occupation. The average age of onset in the current KC cohort was 20 years. Patients with a positive family history and those who had allergy resulting in eye rubbing but who also had a family history of KC were included in the study. Patients with eye trauma, other corneal pathology where KC was secondary to infection or excessive eye rubbing were excluded from the study.

All the patients were subjected to corneal examination. The average Orb scan measurements (curvature of the anterior and posterior corneal layer) of the KC patients was 438.6±47.7 μm (normal range~550 μm), suggesting thinning of the cornea. The progression of the disease based on corneal thinning was measured by corneal pachymetry, the patients had thickness of 462.5 μm±8.0 (normal range~554.9 μm±7.4). Keratometry was performed to measure the curvature of the anterior surface of the cornea, patients had a curvature of 52.5±8.8D (normal range 7.2±0.8D). Age and sex matched 100 (males=62, females=38) healthy control individuals were also randomly selected and were negative for any eye disease, had no other major health problem and were also negative for any other inherited disease. Blood samples of the individuals were collected after obtaining informed written consent.

The genomic DNA was extracted from the lymphocytes using the salting-out method. Briefly the protocol consisted of lysis of red blood cells by 1X erythrocyte lysis buffer (ELB) followed by lysis of white blood cells with Tris-Natrium chloride-EDTA buffer (TNE), SDS, and Pronase E. After an overnight incubation of the samples at room temperature the DNA was salted out using NaCl and ethanol and the precipitated DNA was re-suspended in TE buffer and stored at -20°C till further use.

Genotyping

The amplification of KC sporadic cases was carried out using the primers and conditions given in **Table 1**. The reaction consisted of 50-70 ng of genomic DNA, 0.5 mM dNTPs, 1X ammonium sulphate Taq buffer, 2.5 U Taq DNA polymerase and GIBCO® water. The thermocycling was carried out at an

initial denaturation for five min at 95 °C, followed by 35 cycles of denaturation at 95 °C for 30 seconds, primer annealing for 30 sec at the respective temperatures given in **Table 1** and chain extension at 72 °C for 30 seconds. The final extension was at 72 °C for seven min.

Restriction Fragment Length Polymorphism Analysis

The amplified products were digested overnight with the respective enzymes (Tru1I (MseI) for rs17501108 and Bg1II for rs3735520) as per the manufacturer's protocol and the digested products were electrophoretically separated on 3% agarose gels and visualized under UV transillumination using a gel documentation system. In order to validate the restriction fragment length polymorphism (RFLP) results 10% samples of each genotype were also sequenced.

Statistical Analysis

Hardy-Weinberg equilibrium of genotype frequencies in the control population was calculated for each SNP of HGF (rs17501108, rs3735520). To assess the association of the disease with genotype, logistic regression analysis was performed while adjusting for gender. The analysis was performed using R software (R Core Team, 2012; R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL <http://www.R-project.org/>). A p-value of <0.05 was taken as statistically significant.

RESULTS

Association Analysis of HGF SNPs

The global allele frequencies for the studied SNPs for rs17501108 alleles is G=0.89, T=0.11, and rs3735520 alleles is T=0.45, C=0.55, respectively. The SNP-based association analysis of HGF (rs17501108, rs3735520) did not show any significant difference in genotype and allele frequency distribution among cases and controls for both the genes. In addition, gender adjusted multivariate logistic regression analysis also did not reveal any disease association of the SNPs (HGF: rs17501108, p>0.05, odds ratio (OR)=1.19[95% confidence interval (CI)=0.55-2.61]; rs3735520, p>0.05, OR=1.58[95% CI=0.70- 3.56]) (**Table 2**).

Table 2. Logistic regression analysis for rs17501108 and rs3735520 in HGF

	Frequencies		Estimate: KC vs controls	z-value	OR (95% CI)	p-value
	Controls (n=100)	KC (n=100)				
Genotype: rs17501108						
GG	82 (82.0%)	88 (88.0%)	0.1769	0.4437	1.1936 (0.5461-2.6085) ^a	0.6572
GT	15 (15.0%)	9 (9.0%)	-	-	-	-
TT	3 (3.0%)	3 (3.0%)	-	-	-	-
Allele frequency						
G	179 (89.5%)	185 (92.5%)	-	-	1.447 (0.688-3.060) ^b	0.383 ^b
T	21 (10.5%)	15 (7.5%)	-	-	-	-

Table 2 (Continued). Logistic regression analysis for rs17501108 and rs3735520 in HGF

	Frequencies		Estimate: KC vs controls	z-value	OR (95% CI)	p-value
	Controls (n=100)	KC (n=100)				
Genotype: rs3735520						
TT	27 (27.0%)	28 (28.0%)	0.4591	1.1091	1.5826 (0.7031-3.5622) ^a	0.2673
CT	49 (49.0%)	54 (54.0%)	0.3213	0.8670	1.3790 (0.6669-2.8515) ^a	0.3859
CC	24 (24.0%)	18 (18.0%)	-	-	-	-
Allele frequency						
T	103 (51.5%)	110 (55.0%)	-	-	1.151 (0.762-1.739) ^b	0.548 ^b
C	97 (48.5%)	90 (45.0%)	-	-	-	-

Note. ^aAge and gender adjusted OR and (95% CI) from multivariate logistic regression analysis & ^bOR and (95% CI) from univariate logistic regression analysis

Table 3. Pairwise linkage disequilibrium (LD) for HGF SNPs

		HGF
		rs3735520
D		0.0086078
D'	rs17501108	0.2155329
Correlation r		0.0655002
χ^2		1.7161089
p-value		0.1901947

Note. D: Raw difference in frequency between the observed number of AB pairs and the expected number; D': Scaled D spanning the range [-1, 1]; r: Correlation coefficient between the markers; χ^2 : Chi-square statistic for linkage disequilibrium, i.e., $H_0: D=D'=r=0$; & p-value: Chi-square p-value for marker independence

Based on the SNPs allele frequencies in the studied population, pairwise linkage disequilibrium (LD) showed that HGF rs1750110 and rs3735520 SNPs were not in LD (**Table 3**).

The combined role of SNPs of HGF was also examined for KC using haplotype analysis for the respective HGF SNPs. The reference haplotypes (due to their higher frequency in both cases and control) for HGF was GT. The haplotype did not reveal a protective or disease-causing role in the development of KC (**Table 4**).

DISCUSSION

KC is an ocular condition for which the underlying genetic etiology is still not well understood. Due to its increasing incidence in young adults, studies are being conducted worldwide to determine the role of genetics in disease development and progression. Present study was designed to investigate the association of KC with HGF and for this purpose case-control association analysis was conducted in south Asian population of Pakistani origin by genotyping SNPs in previously reported associated gene HGF. Present study came with the findings that although globally an association exist between HGF and KC but genotyping data of Asian population of Pakistan origin does not show any association between HGF and pathogenesis of KC.

Polymorphisms in different genes are reported to be a risk factor in the development of KC worldwide, but only a few studies have been conducted on determining the association of HGF SNPs with KC. It was recently suggested the involvement of SNPs rs3735520 and rs17501108, residing upstream in the promoter region of HGF, in the development of KC in Caucasians [16]. However, the findings of the current study in south Asian patients of Pakistani origin are not in cohesion with the previously demonstrated association of HGF SNPs in the pathogenesis of KC. It is possible that due to the genetic heterogeneity in KC there is a possibility that there is

Table 4. Haplotype analysis of HGF SNPs

Haplotype	Estimate	z-value	OR (95% CI)	p-value
HGF: rs17501108 & rs3735520				
GC	-0.1566	-0.6857	0.8550 (0.5464-1.3378) ^a	0.4937
TC	-0.5868	-1.0146	0.5560 (0.1789-1.7277) ^a	0.3115
TT	0.2346	0.3154	1.2644 (0.2942-5.4344) ^a	0.7527
GT	-	-	-	-

Note. ^aAge and gender adjusted OR and (95% CI) from multivariate logistic regression analysis & The reference haplotype for HGF is GT

some other underlying genetic or environmental factor, which might be contributing to the development of the phenotype in patient of Pakistan origin. In addition, the studied cohort size is relatively small, which seems to be one of the limitations of the study. Therefore, replication study of the studied HGF SNPs is suggested on larger cohort of KC patients, not only of Pakistani population, but different ethnicities of South Asia.

KC is heterogenous disorder, which is believed to be caused by genetic and environmental factors [5] and apparently it seems to be unidirectional to focus only genetic part and ignoring the environmental factors. Although some other factors are also involved in the pathogenesis of KC like rubbing of eye [16], allergy [17], and contact lens wear [18] but some family history genetics have been linked to play a dominant role in the pathogenesis of KC [19]. However, ethnic differences have been found vary widely in the prevalence of KC. The reports of two surveys in the UK indicated a prevalence 4.4 and 7.5 times greater for Asian (Bangladeshi, Indian, and Pakistani) subjects compared with white Caucasians [20, 21]. One of the factor for genetic association of KC is consanguineous relations, especially first cousin marriages, which commonly take place in the Asian population of the area assessed [22]. Although present study, took the sample of KC patients and tried to find the association of HGF with KC but it would be interesting to know the association of cousin marriage in KC patients, which may help us to conclude the association of cousin marriage in the pathogenesis of the KC. However, this link of association of consanguineous relations in KC remain limitation of the study. Keeping in view, we can propose that globally HGF might be involve in the pathogenesis of KC but in Pakistani population this gene is present insignificant association with KC.

CONCLUSION

Present study came with the findings that although globally an association exist between HGF and KC but genotyping data of Asian population of Pakistan origin does not show any association between HGF and pathogenesis of KC.

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Ethical statement: The author stated that the study conformed to the Helsinki Declaration and was approved by Ethics Committee/Institutional Review Board of College of Pharmacy, University of Hafar Al Batin on December 8, 2021 with approval code: 0015-1443-S. Informed consents were obtained from the participants. Personal information was kept confidential.

Declaration of interest: No conflict of interest is declared by the author.

Data sharing statement: Data supporting the findings and conclusions are available upon request from the author.

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