

Association of hepatocyte growth factor gene polymorphisms with primary angle closure glaucoma from Lahore, Pakistan

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Abstract

Objective: To determine the association of hepatocyte growth factor gene single nucleotide polymorphisms rs5745718 and rs17427817 with primary angle closure glaucoma.

Methods: This case-control study was conducted at the Department of Biotechnology, Lahore College for Women University, Lahore, Pakistan, from August 2016 to September 2017. In this study seventy sporadic cases of primary angle closure glaucoma and sixty healthy controls were enrolled from different hospitals of the Lahore, Punjab. Blood samples were obtained from all the subjects. Genomic deoxyribonucleic acid was extracted by non-organic method. Two single nucleotide polymorphism (SNPs) rs5745718 and rs17427817 in Hepatocytes Growth Factor (HGF) gene were genotyped in patients and ethnically same healthy controls by using polymerase chain reaction restriction fragment length polymorphism (RFLP) assay. Data was analysed using SPSS (version 20).

Results: Of the 130 subjects, 70 (54%) were cases and 60 (46%) controls. The mean age of the cases was 54±17 years (range: 13-85 years). The differences in genotype distribution were statistically significant for rs 5745718 ($p=0.005$ and $p=0.009$), while results were not significant for rs 17427817 ($p=0.06$ and $p=0.09$) between the cases and the controls.

Conclusion: AC alleles were found to be protective while CC alleles were a risk factor for primary angle closure glaucoma in rs5745718 single nucleotide polymorphism.

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Introduction

Glaucoma is heterogeneous disorder and both environmental as well as genetic factors play an important role in its pathogenesis.¹ Glaucoma is considered to be the second leading cause of blindness worldwide. The incidence of blindness in Pakistan is 2.7% according to the National Health Survey of 2003 and in Pakistan glaucoma is the fourth most common cause of blindness.² Primary angle closure glaucoma (PACG) shows characteristic shallow anterior chamber, complete or partial chamber angle closure, increased thickness of the lens, hyperopic refractive error and short axial length. In Pakistan, chronic angle closure glaucoma (CAC) glaucoma (16.2%) is the common type after open angle glaucoma (47.3%).³

A genetic study revealed that many genes are associated in PACG development. The first human locus associated with CAC glaucoma and nanophthalmos (small eyes) phenotype is NNO1.⁴ Genome-wide association studies (GWAS) have recognised three susceptibility loci

(rs110224102 in PLEKHA7; rs3753841 in COL11A1, and rs1015213 located between PCMTD1 and ST18 on chromosome 8q) for PACG.⁵ Association studies also proposed the contribution of numerous single nucleotide polymorphisms (SNPs) of the matrix metalloproteinase-9 (MMP-9) gene,⁶ membrane type frizzled related protein (MFRP) gene,⁷ methylenetetrahydrofolate reductase gene (MTHFR),⁸ retinal homeobox gene (CHX10),⁹ ABCG5 gene,¹⁰ secreted protein acidic and rich in cysteine (SPARC) gene,¹¹ calcitonin receptor-like receptor (CALCRL) gene,¹² superoxide dismutase (SOD2) gene and hepatocyte growth factor (HGF) gene.¹³ In Pakistan, variations in the heat-shock protein 70 (HSP70), endothelial nitric oxide synthase (eNOS), association of polymorphisms in MMP1, MMP9, MTHFR gene, C677T, A1298C and PACG and primary open angle glaucoma (POAG) have been studied.^{6,8,14} Previously, it was reported that mitochondrial deoxyribonucleic acid (DNA) haplo groups were in higher frequency in PACG patients in Saudi Arabia, suggesting their possible role in PACG.¹⁵ In Indian population, CYP1B1 mutations shared similar haplotypes background both in POAG and PACG phenotypes.¹⁶ However, no mutations in the POAG genes, such as myocilin (MYOC), optineurin (OPTN), and WDR36 have been identified in PACG patients.¹⁷

The HGF gene played a significant role in the

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emmetropisation process of the eye. HGF has a heterodimer molecule consisting of 34-kDa α -subunit and 69-kDa β -subunit.¹⁸ HGF is produced by stromal cells, stimulates proliferation of epithelial cell, motility, morphogenesis and angiogenesis in several organs via tyrosine phosphorylation of its receptor, c-Met. The gene that encodes the human HGF consists of 18 exons and 17 introns of about 70kb and located on chromosome 7q11.1-21.¹⁹

In a study in Nepal, 12 tag SNPs were selected and genotyped to study the common variations in the HGF gene. Four HGF SNPs rs5745718, rs12536657, rs12540393 and rs17427817 were seen to be significantly associated with PACG.²⁰ Three SNPs, rs17427817, rs5745718 and rs3735520 in the HGF gene were genotyped by restriction fragment length polymorphism and it was proposed that rs5745718 and rs17427817 were associated with a decreased risk of PACG in the Chinese population.²¹

The current study was planned to investigate whether this polymorphism is a risk factor for PACG in the Pakistani population.

Patients and Methods

The case-control study was conducted at the Department of Biotechnology, Lahore College for Women University, Lahore, Pakistan, from August 2016 to September 2017, and comprised PACG patients and healthy controls enrolled from different hospitals of the Lahore. After approval from the institutional review committee, the sample size was calculated using the 1:1 distribution of cases and controls.²² Permission was also obtained from all the hospitals from which the subjects were enrolled, while written informed consent was obtained from all the subjects and parents in case of children.

Those included were PACG patients on the basis of existence of glaucomatous optic neuropathy and defects in the visual field by expert ophthalmologist of the hospital and confirmation was done in these patients after performing different tests which included tonometry, pachymetry, gonioscopy, cup-to-disc (C/D) ratio measurement and retinal nerve fibre layer examination. Normal healthy subjects with no history of glaucoma were enrolled as the controls.

Blood samples 5-10ml was collected in vacutainer purple capped tubes (Becton, Dickinson and Company, NJ, USA). Total genomic DNA was extracted through non-organic method which involves cell lysis, proteinase K digestion and use of salt extraction for the precipitation of contaminating proteins.²³ The quantity of the DNA in the sample was observed on agarose gel by comparing the

intensity of the band with the known standard DNA samples. Polymerase chain reaction (PCR) was used to amplify the target DNA in the HGF gene. The forward and reverse primers were: 5' CTACAAAGAACCTACAACACAA3' and 5'TCATCTTACTGAACTTGTTT3' for SNP rs5745718 and 5'TCCCTCGGGATTGAGAACAGTG3' and 5'TTTGGAGGTCTTTACTACTT 3' for SNP rs17427817, respectively.²¹ The PCR programmes (T100 thermal cycler Bio - Rad, Richmond, USA) used comprised an initial denaturation at 94°C for 2 minutes followed by 35 cycles of denaturation at 94°C for 45 seconds, annealing at (54°C for rs17427817 and 52°C for rs5745718) for 45 seconds and extension at 72°C for 45 seconds. After the last cycle, the reaction was held at 72°C for 10 minutes. PCRs were carried out in a total volume of 25 μ l and contained 2 μ l of genomic DNA. The PCR products of rs17427817 and rs5745718 polymorphisms were respectively digested with restriction enzymes NlaIV and BssECI (Thermo fisher Scientific, USA). NlaIV [*Neisseria lactamica* restriction endonuclease IV] is restriction enzyme which recognises the sequence GGNNCC. While BssECI (*Geobacillus stearothermophilus* EC) cuts the sequence at recognition site of CCNNGG. Reaction was prepared by mixing 10 μ l PCR product, 1 μ l restriction enzymes (NlaIV, BssECI) 18 μ l nuclease free water, and 2 μ l Tango buffer for each reaction mixture and incubated at 37°C and 55°C for 3 hours for NlaIV, BssECI enzymes respectively. The bands were separated on 2% agarose gel electrophoresis. Afterwards ultraviolet (UV) trans-illuminator was used to observe the digestion products. SPSS 20 was used for data analysis. Level of significance was set at 5% for determining statistical significance. Pearson chi-square and fisher exact tests and odd ratios (OR) with 95% confidence intervals (CI) were calculated to check the strength of association between HGF polymorphisms and PACG risk.

Results

Of the 130 subjects, 70(54%) were cases and 60(46%) controls. The mean age of the cases was 54 $\pm$ 17 years (range: 13-85 years). Their eyes had enlarged globes and

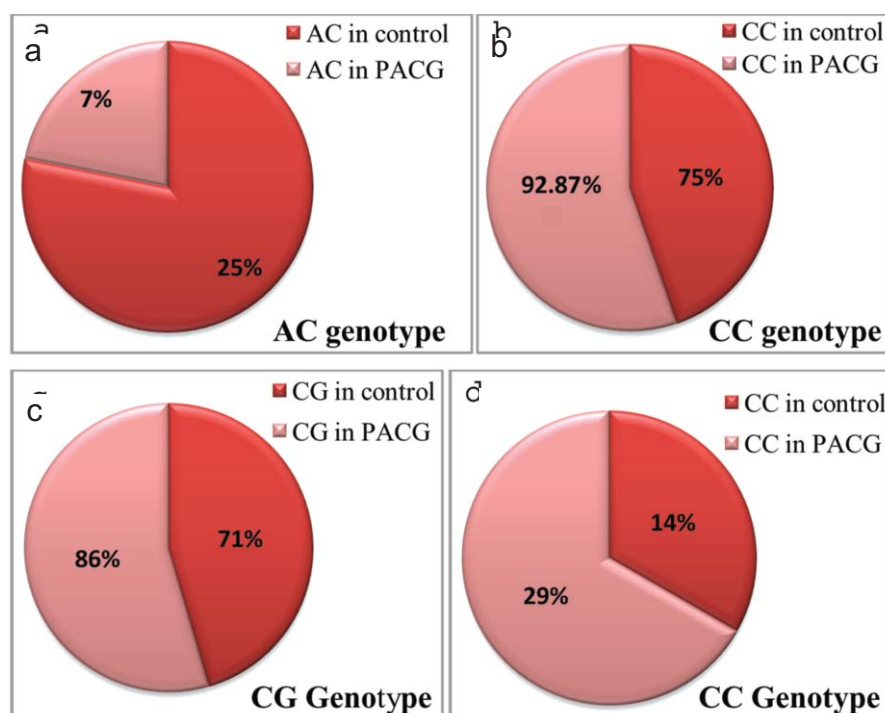
Table-1: History and clinical features of Primary Angle Closure Glaucoma (PACG) patients.

Characteristics	Female (N=33)	Male (N=37)
Mean Age (years)	(56 $\pm$ 13.6)	(54.8 $\pm$ 16.9)
Mean IOP mmHg	(27 $\pm$ 13.6)	(39 $\pm$ 14.54)
Hyperopia	10(7%)	8(5.6%)
Watery and reddish eyes	10(7%)	16(11.2%)
Cataract	5(3.5%)	6(4.2%)
Ocular redness and blurred vision	6(4.2%)	7(4.9%)

IOP: Intra Ocular Pressure.

Table-2: Genotypic frequencies in Primary Angle Closure Glaucoma (PACG) patients and controls for Hepatocyte Growth Factor (HGF) Single Nucleotide Polymorphisms (SNP) (rs5745718) and (rs17427817) in different populations.

SNPs	Countries	Genotypes	Control %	PACG%	References
rs5745718	China	AC	14.60%	25%	Jiang et al., 2013 ²¹
		CC	84%	70.60%	
	Nepal	AC	15%	26%	Awadalla et al., 2011 ²⁰
		CC	84%	70%	
	Pakistan	AC	25%	7%	In the present study
		CC	75%	92.87%	
rs17427817	China	AA	-	-	Jiang et al., 2013 ²¹
		CG	18.8%	28.6%	
	Nepal	CC	1.4%	4.6%	Awadalla et al., 2011 ²⁰
		CG	18%	32%	
	Pakistan	CC	2%	5%	In the present study
		CG	71.42%	85.71%	
CC		28.57%	14.28%		
GG		-	-		

**Figure:** (a and b) Pie graphs showing the significant genotypic difference between controls and Primary Angle Closure Glaucoma (PACG) patients (rs5745718); and (c and d) showing the non-significant genotypic difference between controls and PACG patients (rs17427817) in Pakistani population.

they felt pain, blurred vision and itching in both eyes. The intraocular pressure (IOP) ranged 5-73mmHg in the patients. The mean age of females was 56 ± 13.6 years (25-85 years) and IOP ranged 4-50mmHg. The mean in males

was 54.8 ± 16.9 years (range: 13-80 years) and IOP ranged 5-80mmHg (Table-1). The demographic characteristics between the PACG cases and the healthy individuals were similar. There was no significant difference between the cases and the controls with respect to age and gender.

Of the 70 cases, 5(7%) carried AC genotype, while 65(93%) had CC genotype. In contrast, 15(25%) controls had AC genotype and 45(75%) had CC genotype. The frequencies of the AC genotype and A allele of rs5745718 were significantly decreased in cases compared to the controls ($p < 0.05$).

In the analysis of rs17427817, 60(85.71%) patients carried CG genotype and 10(14.28%) had CC genotype. Among the controls, 43(71.42%) had CG genotype and 17(28.57%) had CC genotype. Non-significantly decreased frequency of CC genotype and C allele of rs17427817 were observed in the patients compared to the controls ($p > 0.05$) (Figure). Genotyping of the HGF gene SNP rs17427817 was not significantly associated

($p > 0.05$), while SNP rs5745718 was significantly associated with PACG ($p < 0.05$) compared to the controls.

Discussion

PACG is a neurodegenerative optic ailment and is characterised by progressive visual field defect, retinal ganglion cells and optic nerve rim loss.² Asians are more affected with PACG than the other races because of an unusually high incidence of PACG among siblings of affected patients, and it has been suggested that genetic factors are involved in its pathology and the action of a large number of grouped or independently inherited genes along with environmental factors result in anatomical abnormalities of PACG.²⁴

In Pakistan, association studies have been done between polymorphisms in MMP1, MMP9, MTHFR genes and PACG and POAG. For the study, sporadic and control samples were collected from two ethnic groups i.e., Pathans (Khyber Pakhtunkhwa province, northern Pakistan) and Punjabis (Punjab province, central Pakistan).

The TT, AC and combined genotype TTAC of MTHFR C677T, A1298C, MMP1 rs1799750 (-16071G/2G) and MMP9 rs17576 polymorphisms were associated with PACG in Pakistani population.^{7,9} Similarly, number of association studies has been conducted in order to check the association of different genes with primary angle glaucoma in different populations. In Australian-Caucasian population, SNPs rs12536657, rs5745718, rs1743, rs4732402, rs12536657, rs10272030 and rs9642131 of HGF gene showed significant association with low/moderate myopia and hypermetropia.²⁵ SNPs rs2075555 and rs2269336 in the gene COL1A1 were associated with increased risk of myopia in a Japanese and Chinese Han populations.²⁶ Collectively, these findings also indicated that PACG and hyperopia share a common pathway in the development of the disease.

Various SNPs of the HGF gene have been associated with hyperopia. Some SNPs of the HGF gene (rs5745718, rs12536657, rs12540393, rs17427817 and rs3735520) were also recognised as being associated with PACG in the Nepalese and Chinese population. Previous studies have stated that after corneal injury the messenger ribonucleic acid (mRNA) level of HGF was greater in the rabbit lacrimal gland, and the level of HGF protein was also significantly increased in the aqueous humor of glaucomatous eyes.²⁷ These findings suggested that the HGF protein might have functional roles in the progress of PACG. Secondly, it has been proposed that the up-regulated expression of a certain gene may be associated with certain SNP alleles in its gene.²⁷

The HGF gene is located on chromosome 7q11.1-21 in humans and its protein has been shown to play an important role in the growth stimulation and movement of several tissues of the human eye. The HGF gene and its receptor c-MET are both known to be expressed in all three cellular layers of the cornea, but its expression is lower in the epithelium than in the stroma and endothelium. The protein is also formed by the lacrimal glands and is found in tears. HGF gene consists of 18 exons and 17 introns of about 70kb. The general organisation of the plasminogen and the HGF gene is very homologous.²⁸

In our study, genotypic frequencies of HGF SNPs rs5745718 and rs17427817 were observed. Results showed that AC genotype and A allele of the rs5745718 was associated with a reduction in the threat of PACG. Frequency of AC genotype in PACG patients was decreased compared to healthy controls. The ratio of the male was more than the female. In China, CC genotype and C allele of the rs5745718 was considered a protective genotype, and in Nepal rs5745718 A allele was the associated risk.^{18,19} Both studies are in contrast to our finding. One of the reasons for difference in frequency may be due to the different ethnic groups. Secondly, it is possible that due to high consanguinity in Pakistan, the frequency of homozygous CC genotype is high in PACG patients.

In SNP rs17427817, the current study observed that the CC genotype and C allele might be associated with a decreased susceptibility towards PACG in the Pakistani population, but it was not statistically significant. Similar studies in China and Nepal reported SNP rs17427817 was significantly associated with PACG^{20,21} (Table-2).

Genetic study of these two SNPs of the HGF gene among PACG patients of Pakistani origin showed conflicting results from other populations which may be due to ethnic differences and environmental factors. Due to limited genetic studies, there is need of further replication studies in different populations and other areas of the country to confirm this association as well as to further explore the role of HGF in the pathogenesis of the disease.

Conclusion

There was significant association of AC genotype and A allele of SNP rs5745718, while non-significant association of CC genotype and C allele of SNP rs17427817 with PACG in Pakistani population.

Disclaimer: None.

Conflict of Interests: None.

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