ORIGINAL ARTICLE

Single nucleotide polymorphism (rs7543472) in EPHA2 gene is associated with age-related cataract in subjects enrolled from Multan in southern Punjab: A case-control study

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Abstract

Objective: To examine whether Ephrin type A receptor 2 gene polymorphisms are associated with susceptibility to age-related cataract.

Methods: The case-control study was conducted from January to May 2014 in Multan, Pakistan, and comprised patients of age-related cataract enrolled from Nishtar Hospital, Multan, and age-matched healthy controls without any type of cataract from the local population. A questionnaire was used to gather clinical and epidemiological data. Deoxyribonucleic acid was extracted from blood samples, and analysis of rs11260867, rs3568293 and rs7543472 single nucleotide polymorphisms was performed by using tetra amplification-refractory mutation system polymerase chain reaction protocol. Data was analysed using SPSS 17.

Results: Of the 230 subjects, 129(%) were patients and 101(%) were controls. Among the three polymorphisms analysed, rs7543472 was associated with age-related cataract. Among the epidemiological and clinical factors, age, diabetes, blood pressure, smoking, radiation exposure, steroids usage and use of tranquilisers were associated with age-related cataract ($p<0.05$ each).

Conclusion: Polymorphism rs7543472 was found to be associated with age-related cataract.

Keywords: Age-related cataract, EPHA2 gene, rs11260867, rs3568293, rs7543472, Tetra ARMS PCR. (JPMA 70: 583; 2020) **https://doi.org/10.5455/JPMA.6232**

Introduction

Cataract is an eye lens problem and it is a major cause of avoidable blindness and visual impairment throughout the world. About 90% of lens protein, including the most abundant and diverse Crystallin protein, are water-soluble and, in order to maintain transparency, they must be present in homogeneous phase and arranged in a uniform order.[1] Alpha crystallin proteins are called molecular chaperones because they prevent the aggregation of proteins, making the lens more resistant to oxidative stress (OS) and aging process.[2] Beta crystallin proteins maintain the transparency of the lens as they interact with other crystallins, keeping them arranged specifically and this protein-protein interaction protects the lens from thermal and oxidative damages.[3] With aging, alpha crystallins lose their chaperone activity and the lens is exposed to thermal and oxidative stress which results in the formation of protein aggregates and cataract formation.[4]

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Age-related cataract (ARC) is multi-factorial disorder with the involvement of numerous genes and environmental risk factors. So far, mutations in at least eight genes are reported to be associated with ATC. Ephrin type A Receptor 2 (EPHA2) gene is reported to be associated with cataract and it is located on chromosome 1p36.13.[5] This gene is composed of 17 exons spanning in a region of 31,73kb and produces a messenger ribonucleic acid (mRNA) of 3970bp, while EPHA2 receptor is composed of 976 amino acid residues and its molecular weight is 130kDa.[6] Multiple mutations in the EPHA2 gene have been recently shown to cause cataracts in humans, contributing to the destabilisation of the receptor and the loss of cell migration activity.[7] EPHA2 regulates lens fibre cells' shape and interactions, and is important for lens transparency development and maintenance.[8]

Cataract is the leading cause of blindness in Pakistan. The fact that cataract is treatable makes it vital that the public becomes aware of its nature and potential problems.[9] In 1994, it was estimated that approximately 500 ophthalmologists had performed 140,000 cataract surgeries in Pakistan, giving a corporate social responsibility (CSR) of 1115/million population per year. The current figure is uncertain because reliable national

evidence is lacking, but the World Health Organisation (WHO) estimates that the CSR has more than doubled to 2400/million population per year.[10] According to a study conducted in 2007, there are approximately 570,000 adults who are blind (<3/60) from cataract in Pakistan, and 3,560,000 eyes with a visual acuity of < 6/60 due to cataract.[11] Studies from southeast Asia are part of literature regarding the single nucleotide polymorphism (SNP) screening in EPHA2 gene and their correlation with ARC in various ethnic groups but there is no specific report on this topic from local Pakistani population.[1,2,9]

The current study was planned to explore the association of SNPs in EPHA2 with ARC. The secondary objective was to document the relationship between ARC and demographic factors.

Materials and Methods

The case-control study was conducted from January to May 2014 in Multan, Pakistan, and comprised patients of age-related cataract enrolled from the Ophthalmology Ward of Nishtar Hospital, Multan, and age-matched healthy controls without any type of cataract from the local population.

After approval from the ethics review committee of the Institute of Molecular Biology and Biotechnology (IMBB), Bahauddin Zakariya University, Multan, the sample was raised, and written informed consent was obtained from all the enrolled subjects before sample and data collection.

A predesigned questionnaire based on risk factors of ARC was used to collect data including age, gender, ethnicity, family history of cataract, smoking habits, alcohol consumption, radiation exposure, hormonal therapy, diabetes and blood pressure of patients.

During clinical evaluation, cataract was confirmed by testing visual acuity (VA) of each subject and then the status of intraocular lens (IOL) was evaluated by slit-lamp biomicroscopy at Ophthalmology Ward of Nishtar Hospital, Multan. Prevalence of four types of cataract was recorded by grading lens opacities classification system using retro illumination technique for cortical and posterior subcapsular and direct focal illumination for nuclear cataract type.

Blood sample (3-5 ml) from each subject was preserved in ethylenediaminetetraacetic acid (EDTA)-coated tubes and stored at -4°C till they were further analysed at the Institute of Molecular Biology and Biotechnology (IMBB), Bahauddin Zakariya University, Multan. Deoxyribonucleic acid (DNA) extraction from blood was carried out by inorganic method.[12] Amplification of target DNA in EPHA2 gene was done by polymerase chain reaction (PCR) using tetra amplification-refractory mutation system (ARMS) PCR. In this method, DNA fragment having polymorphism was amplified by using four primers. The primers were designed in such a way that PCR products after amplification represented different sizes for each allele and could be easily resolved by agarose gel electrophoresis. Three PCR products were for heterozygous genotypes, and two PCR products represented homozygous genotype. Oligonucleotide primers were used for genotyping of targeted DNA (Annexure-A).

Annexure-A: Oligonucleotide primers sequences designed and used for the amplification of single nucleotide polymorphism (SNP) in single nucleotide polymorphisms Ephrin type A receptor 2 (EPHA2) gene.

rs3768293	
Forward inner primer (G allele)	AAATAGCTCTCTCCTCCACCAGATCTATG
Reverse inner primer (T allele)	CCCAGCTTTCTGGAGTCTCAGTTTTATTA
Forward outer primer (5' - 3')	AGCTGTGGGTCTATGTGTTGTGGAG
Reverse outer primer (5' - 3')	CCTGGGCTGTGGGGGTTTAT
rs11260867	
Forward inner primer (C allele)	TAGCATTCTGGATGCCCACAGAC
Reverse inner primer (G allele)	GGGGGAGTCCGGATGACAC
Forward outer primer (5' - 3')	CCTGAAACTTTGGCCTCCCC
Reverse outer primer (5' - 3')	CTTCTCCCAGGTGTCCCTGAGTC
rs7543472	
Forward inner primer (C allele)	CGCCACTGCCCTCTAGCATC
Reverse inner primer (T allele)	TTTTTGAGACGAGTCTCGCTCTATTAACA
Forward outer primer (5' - 3')	TGATCCAAAAAATCAGTGACAGGCT
Reverse outer primer (5' - 3')	ACCACCACATCCGGTTAATTTTTGT

PCR was performed in a final reaction volume of 25 l containing 1X Taq Buffer, 0.2mM deoxynucleotide (dNTP) mixture, 1.6mM magnesium chloride, 2.5U/μl Taq polymerase (Fermentas, UK), 0.5μl of each outer and 1μl of each inner primer (10 picomol), 10 nano-gram of DNA template and deoxyribonuclease (DNase) free deionised water. For the negative control, water was used instead of DNA.

DNA amplification was carried out in a DNA thermal cycler (Gene Amp PCR system 2700 Applied Biosystems Inc., UK). The thermo-profile used consisted of initial denaturation carried out at 95°C for 4min followed by 30 cycles of denaturation at 94°C for 30s, annealing at 50°C for 30s, elongation at 72°C for 45s and final extension was carried out at 72°C for 5min. PCR products were held at 4oC until separated by electrophoresis on a 2.5% agarose gel and visualised under an ultraviolet (UV) trans-illuminator (Biostep, Germany).

Data was analysed using SPSS 17. Association between SNP genotypes and cataract or type of cataract was

determined by using cross-tabulation and statistical significance of it was investigated by chi square tst. Binary logistic regression was applied to find any association between ARC and all the risk factors and to demonstrate the effect of each parameter in controls and patients. Pearson and Hosmer-Lemeshow tests were applied as goodness of fit test for binary logistic regression analysis. P<0.05 was considered significant.

Results

Of the 230 subjects, 129(%) were patients and 101(%) were controls. Among the cataract types, mixed cataract was the most common, followed by nuclear, cortical and posterior subcapsular tupes (Table-1).

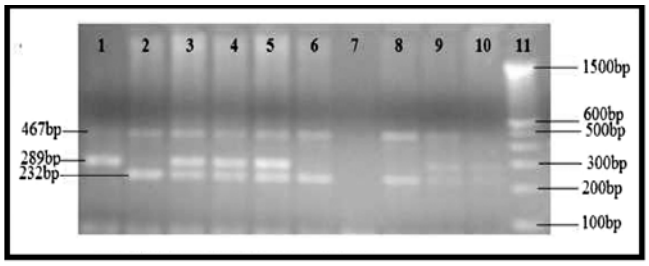

Figure-1: Ehidium bromide-stained electrophorosed gel PCR products. 100bp ladder (lane 11); Age-related cataract-patients showing the homozygous wild GG genotype (lanes 1, 6, 8); heterozygous G/T genotype (lanes3, 4, 5, 9, 10); homozygous mutant genotype TT (lane 1).

In rs7543472, wild type genotype frequency was higher in controls than cataract patients and mutant genotype frequency was higher in cataract patients than controls (p<0.001) (Figure-1).

Wild type and mutant allele of rs3768293 were distributed equally in cases and controls (P=0.154; Figure-2).

With respect to rs11260867, only 1(0.8%) case was heterozygote while the remaining had wild type genotype. All controls carried wild type genotype. There was no heterozygous and homozygous mutant genotype among the controls. Overall, there was not a single individual with

Annexure-B: Goodness of fit tests for binary regression analysis to account for the correlation between studied demographic, clinical variables and studied single nucleotide polymorphisms (SNPs) in Ephrin type A receptor 2 (EPHA2) gene with age-related cataract.

Tests	DF	Chi Square	P value
Pearson	218	229.95	0.276
Hosmer-Lemeshow	8	6.82	0.556

Table-1: Prevalence of different cataract types among the enrolled subjects. males and females. Parenthesis shows % prevalence for each gender.

Gender	Cataract type				Total
	Nuclear cataract	Cortical cataract	Posterior sub-capsular cataract	Mixed cataract	
Male	16 (30.7%)	6 (11.5%)	4 (7.7%)	26 (50%)	52
Female	23 (29.8%)	3 (3.9%)	2 (2.6%)	49 (63.6%)	77
Total	39	9	6	75	129

Table-2: Genotypic and allelic frequency of three single nucleotide polymorphisms (SNPs) in single nucleotide polymorphisms Ephrin type A receptor 2 (EPHA2) gene in enrolled case and control subjects. P values indicates the result of Chi square test. Parenthesis shows the % prevalence of each genotype and allele.

SNP	Genotype Allele	Cases (n=129)	Controls (n=101)	Chi Square value	P-value
	CC	33(25%)	50 (49.5%)		
	C/T	81(63%)	50(49.5%)	19.95	P < 0.001***
rs7543472	TT	15(12%)	1(0.009%)		
	C	147(57%)	150(74%)	14.78	P < 0.001***
	T	111(43%)	52(26%)		
	GG	33(25%)	18 (18%)		
	G/T	66(51%)	58(57%)	3.74	0.154
rs3768293	TT	26(20%)	25(26%)		
	G	140(54%)	94(46%)	2.708	0.099
	T	118(46%)	108(56%)		
	CC	128 (99%)	101(100%)		
	C/G	1 (1%)	0(0%)	0.77	0.63
rs11260867	GG	0(0%)	0(0%)		
	C	257(99.6%)	200(100%)	0.77	0.37
	G	1(0.4%)	0(0%)		

P > 0.05 = Non significant; P < 0.001 = Highly significant (***).

Table-3: Association of single nucleotide polymorphisms (SNPs) of single nucleotide polymorphisms Ephrin type A receptor 2 (EPHA2) gene with different types of age-related cataract.

	Genotype and odd ratios	Nuclear (n=104)	Cortical (n=74)	Posterior subcapsular (n=16)	Controls (n=101)
	Genotype				
	CC	25(28%)	18(44%)	4(50%)	50
rs7543472	C/T	65(53%)	48(44%)	12(50%)	50
	TT	14(17%)	8(2%)	0(0%)	1
	P-value	0.962	0.005**	0.002**	
	Odd Ratio(95% CI)	2.6(1.42-4.76)	2.67(1.37-5.2)	1(0.19-5.19)	
	Genotype				
	GG	28	16	8	18
	G/T	56	47	3	58
rs3768293	TT	20	11	5	25
	P-value	0.395	0.017*	0.27	
	Odd Ratio(95% CI)	0.62(0.31-1.25)	0.91 (0.4-1. 8)	0.12(0.03-0.49)	
	Genotype				
	CC	103	73	16	101
	C/G	1	1	0	0
rs11260867	GG	0	0	0	0
	P-value	0.07	0.07	0.07	
	Odd Ratio (95% CI)	NA	NA	NA	

NA = Not available
P > 0.05 = Non significant; P < 0.05 = Least significant (*); P = 0.01 = Significant (**)
CI: Confidence interval.

Table-4: Association of different haplotypes with age-related cataract. P value represents the results of Chi square test.

rs7543472	rs3768293	rs11260293	Affected	Normal	Total (%)	Chi Square / P-value	Likelihood ratio
CC	GG	CC	9	9	18(8%)	.210/.664NS	.663
	G/T	CC	18	29	47(20%)	1.128/.274NS	.273
	TT	CC	6	13	19(9%)	2.111/.180NS	.176
C/T	GG	CC	24	9	33(14%)	8.500/.004**	.003
	G/T	CC	39	28	67(29%)	2.036/.113NS	.112
		C/G	1	0	1(0.4%)	Cannot be calculated	
	TT	CC	16	12	28(12%)	.521/.477NS	.477
TT	GG	CC	4	0	4(1.7%)	Cannot be calculated	
	G/T	CC	7	1	8(3%)	5.355/.04*	.030
	TT	CC	4	0	4(1.7%)	Cannot be calculated	

P > 0.05 = Non significant; P < 0.05 = Least significant (*).

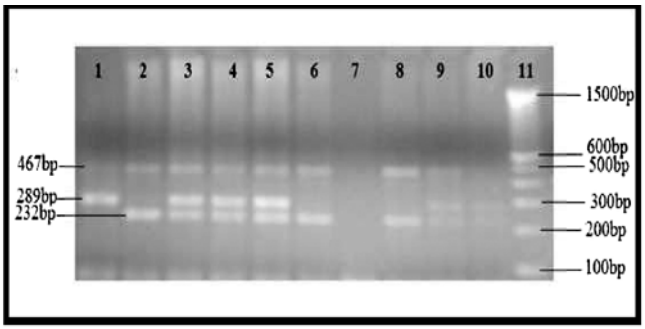

Figure-2: Ehidium bromide-stained electrophorosed gel PCR products. 100bp ladder (lane 11); ARC-patients showing the homozygous wild GG genotype (lanes 2, 6, 8); heterozygous G/T genotype (lanes 3, 4, 5, 9, 10); homozygous mutant genotype TT (lane 1) for EPHA2 rs3768293.

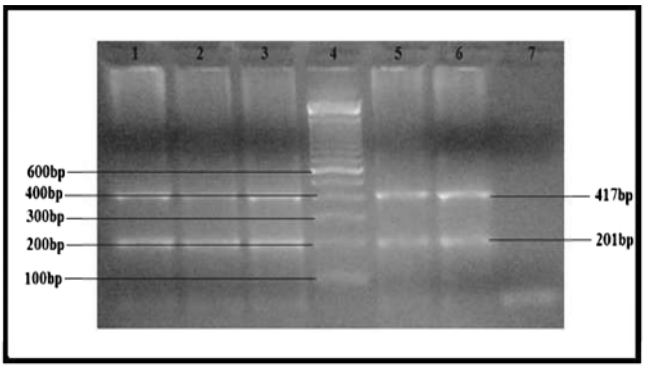

Figure-3: Ehidium bromide-stained electrophorosed gel PCR products. 100bp ladder (lane 1); ARC-patients showing the homozygous wild CC genotype (lanes 1, 2, 5, 6); negative control (lanes 7); for EPHA2 rs11260867.

Table-5: Distribution of different demographic factors in affected and normal subjects. P value represent the result of Chi square test.

Risk factor	Category	Control	Case	Chi square	P Value
Age (years)	<50	53(52%)	25(19%)	39.33	P < 0.001***
	51-60	42(41%)	66(51%)		
	61-70	5(4.9%)	31(24%)		
	71-80	0(0%)	5(4%)		
	>80	0(0%)	2(1.5%)		
Family history of cataract	Yes	0(0%)	1(0.77%)	0.786	0.561
	No	101(100%)	128(99.3%)		
History of diabetes	Yes	5(5%)	21(16%)	7.25	0.007 **
	No	101(95%)	108(84%)		
History of blood pressure	Yes	7(7%)	29(22%)	10.37	0.001***
	No	101(93%)	100(78%)		
Vitamins use	Yes	3(3%)	5(1.5%)	1.58	0.313
	No	101(100%)	127(98.5%)		
Steroids use	Yes	0(0%)	15(11%)	12.56	0.001***
	No	101(100%)	114(89%)		
Hormones intake	Yes	0(0%)	0(0%)	-	-
	No	101(100%)	129(100%)		
Alcohol intake	Yes	0(0%)	1(0.77%)	0.786	0.561
	No	101(100%)	128(99.3%)		
Tranquilizers use	Yes	5(5%)	10(8%)	8.185	0.003 **
	No	101(100%)	119(92%)		
Smoking history	Yes	2(1.99%)	27(21%)	18.461	0.001***
	No	99(98%)	102(79%)		
Radiation exposure	Yes	3(3%)	15(11%)	5.886	0.015*
	No	101(97%)	114(89%)		

P > 0.05 = Non significant; P < 0.05 = Least significant (*); P < 0.01 = Significant (***); P < 0.001 = Highly significant (***).

Table-6: Comparative frequency distribution of Ephrin type A receptor 2 (EPHA2) polymorphisms rs11260867, rs 3768293, rs754347 in various populations.

Population/ Country	Sample (N)	rs11260867		rs3768293		rs7543472		Reference
		C %	G %	G %	T %	T %	C %	
Southern Punjab/Pakistan	230	100	0	55	45	34	66	Present study
India	7474	7	93	NA	NA	21	89	Sundareson et al. 2012
Caucasian/North America	174	81	19	40	60	75	24	
Los Angeles	77	95	5	46	54	78	22	http://www.ncbi.nlm.nih.gov/projects/
Gujrati Indians/Texas	88	NA	NA	NA	NA	77	23	SNP/snp_ref.cgi?rs=3768293
Afican Americans/ SW USA	83	94	6	53	47	63	36	
Japanese/ Tokyo	89	100	0	11	89	97	3	http://www.ncbi.nlm.nih.gov/projects/
Hans Chinese/Beijing	86	99	1	11	89	99	1	SNP/snp_ref.cgi?rs=11260867
Chinese/Colorado	170	100	0	17	84	97	3	
Toscans/Italy	176	88	12	47	53	85	15	http://www.ncbi.nlm.nih.gov/projects/
Maasai/Kenya	286	NA	NA	NA	NA	52	48	SNP/snp_ref.cgi?rs=7543472
Luhya/Kenya	180	90	10	54	46	55	45	
Yoruba/Nigeria	226	97	3	53	47	54	46	
Colombians/Colombia	177	90	10	60	40	83	17	
Peurto Ricans/ America	266	81	19	54	46	66	34	
Finnish	184	87	13	34	66	84	16	
British/England	178	82	18	47	53	79	21	
Iberian/ Spain	110	71	29	54	46	68	32	

homozygous mutant genotype. It was clear that rs11260867 had no association with ARC (Table-2; Figure-3).

Odds ratio (OR) for rs11260867 could not be calculated because only one heterozygote was present in mixed type cataract and no heterozygote in all other types of cataract, and no rare homozygote was found in any cataract phenotype (Table-3).

Among all haplotypes for the three SNPs, two haplotypes (CC CC GT and CC CT TT) had significant association with ARC ($p<0.05$) (Table-4). Chi square values for two haplotypes (CC CC TT and CC TT TT) could not be calculated due to rarity of TT homozygote in cases and controls.

Of the 11 demographic risk factors studied, 7(63%) were significantly associated with ARC (Table-5).

Both Pearson and Hosmer-Lemeshow tests indicated the goodness of applied binary regression analysis (Annexure-B).

Discussion

ARC is a growing global public health problem that affects approximately 37 million people and accounts for 48% of all cases of blindness.[13] According to the location of the opacity within the lens, ARC can be classified as cortical (C), nuclear (N), posterior subcapsular (PSC) or mixed (M). The development of ARC can be influenced by multiple factors, ranging from degenerative processes or personal characteristics to environmental and dietary factors. Age, gender, smoking, and exposure to sunlight are the documented risk factors for ARC, and several recent studies have identified numerous SNPs in genes.[14]

EPHA2 belongs to the tyrosine kinase family, and EPHA2 is epithelial cell kinase that interacts with membrane-bound ephrin ligands, which play an important role in morphogenesis and in numerous developmental processes.[15] Structurally, these proteins contain a ligand-binding domain, epidermal growth factor-like domain, and tyrosine kinase catalytic domain.[16] Ephrin-A5, a ligand of EPHA2, features as a regulator for EPHA2, and lack of ephrin-A5 function caused cataract by interfering with lens fibre cell packing in rats.[17] Furthermore, cataractogenesis in EPHA2 knockout mice indicated that EPHA2 was important for keeping lens clarity.[18] In addition, genetic variants of EPHA2 gene were found to be associated with ARC in Caucasian, Indian, Hans Chinese and Italian populations.[19-21] It has also been reported that the TT genotype of rs7543472 was associated with ~2× increased risk for cataracts in different ethnical and geographical populations.[22] it is interesting that the two SNPs in the EPHA2 region most associated with ARC, rs7543472 and rs11260867, lie outside the coding region near the 3′-end of the gene, which has been shown to harbour highly conserved translational control sequences that are believed to facilitate an RNA-based post-transcriptional mechanism for localised regulation of gene expression within cells.[23] These studies of different populations motivated the current study to investigate the association of EPHA2 variants with ARC in southern Punjab population.

It has been reported that Asian populations has a higher prevalence and earlier age of cataract onset than Europeans.[24] Despite the large number of cataract patients, limited studies have been reported from Pakistan regarding the genetic background of cataract, especially ARC. Mutations in EPHA2 have been shown to underlie inherited forms of cataract. Recently, a study reported that the SNPs in DNA repair genes X-ray repair cross-complementary-1 (XRCC1) [Arg194Trp (rs1799782)] and xerodermapigmentosa complementation group D (XPD) [Lys751Gln (rs13181] have no association with the ARC incidence.[25] Another study sequenced EPHA2 gene bi-directionally in subjects having nuclear cataracts, and identified missense mutation c.2353G>A, which resulted in an alanine to threonine substitution (p.A785T).[16]

Among the three SNPs studied in the current research, rs7543472 showed a significant association with ARC in the target population. The finding is in line with studies conducted in Indian[19] and Italian populations.[21] In the population of southern Punjab, allelic frequency for T allele (26%) was less than that of C allele (57%), so T allele was the rare allele. In Indian population, similar results were found but allelic frequencies of Italian population were considerably different as the T allele (77%) was the common allele, indicating race-specific polymorphisms. Frequency of risk allele in the population of southern Punjab was also different from other populations described in International HapMap project.[26] This difference in frequency of T allele from HapMap documentation for Utah Europeans and Gujarati Indians from Texas from Southern Punjab population shows its distinction from other racial groups. Although distribution frequency of risk allele in two African population groups, Nigerian Yoruba and Kenyan Masai, was lower than that of Italian population, it was significantly higher than the risk allele frequency of southern Punjab population, indicating the fact that the population of southern Punjab is distinctive from both African population groups (Table-6).

In the present study, rs3768293 did not show association

with ARC. Genotypic and allelic frequencies showed that both alleles were equally distributed among cataract patients and controls in the population of southern Punjab. Our findings are supported by a study conducted on Hans Chinese population in which 5 SNPs, rs7584209, rs3768293, rs6603867, rs667816 and rs3754334 of EPHA2 gene were studied and two of them, rs7548209 and rs477558, had significant association with age-related lens opacities.[20] In that study, T was the common allele and G was the rare allele, while in the southern Punjab population, both alleles were equally distributed. In a study on three different Caucasian populations, it was reported that rs3768293 had strong association with ARC (p=0.003) and G allele was the risk allele.[18]

Different genotypes of rs3768293 were significantly associated with cortical cataract. For rs7543472, our results overlap the values in the Italian cohort[21] and are higher than the Indian population.[19] Genotypes of SNP rs3568293 showed a significant association with cortical cataract. These results are in accordance with findings in the Caucasian population, but contradictory to those in the Hans Chinese population.[19,20]

Our results indicated that age was significantly associated with ARC. It has been reported in Taiwani population that the highest prevalence of ARC was observed in patients aged 50-60 years.[27] This increased susceptibility to ARC with age is perhaps due to the fact that people of increasing age are most susceptible to diseases due to many factors, such as physical and environmental factors, deterioration in the damage repair system, and genetic predisposition.[28] Our results of binary regression analysis indicated that age, family history of cataract, diabetes, blood pressure, tranquilizers usage and smoking as well as radiation exposure and steroids had a significant association with ARC. These results are in partial agreement with Khosa et al.[25] as it had also reported the association of tranquilizers use, smoking history and steroids use with ARC in Pakistani Punjabi population.

The present study found that majority of ARC patients were females across all the studied age group. This higher incidence of disease in females is probably due to hormonal influence as females have a more complex reproductive physiology than males.[29] High frequency of cataract in females of increasing age in different population has been reported earlier as well.[30] Our results are contradictory to one study which reported ARC to be more prevalent in males than females in Maharashtra.[31]

Conclusion

Polymorphisms rs7543472 was found to be associated with ARC in southern Punjab population.

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Conflict of Interest: None.

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