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Evaluation of thrombospondin–1 gene polymorphisms in corneal allograft rejection in Asian Indian patients

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Purpose: To evaluate the frequency and the association of Thrombospondin 1 (*THBS1*) gene single nucleotide polymorphisms (SNPs) in Asian Indian patients with optical full thickness corneal grafting surgery. **Methods:** Prospective case–control analysis of optical penetrating keratoplasty patients with and without immune rejection and controls for genotyping of 3 *THBS1* gene SNPs (rs1478604 A>G; rs2228261 C>T; rs2228262 A>G) by Amplification Refractory Mutation System-Polymerase Chain Reaction (ARMS PCR). **Results:** Among 58 patients [45 with immune allograft rejection (DNA isolation was possible in 38 samples) and 13 without immune corneal allograft rejection] and 65 controls, allele frequencies observed for rs1478604 (A>G) are A: 69.7% and 72.6%, G: 30.2% and 27.3%; for rs2228261 (C>T) are T: 70.2% and 62.3%, C: 29.7% and 37.6%; and for rs2228262 (A>G) A: 97.4% and 98.4%; G 2.5% and 1.5% respectively. Genotype frequencies were rs1478604 (A>G) AA: 57.8% and 59.3%, AG 23.6% and 26.5%; GG 18.4% and 14%; for rs2228261 (C>T) TT: 40.5% and 33.8%, TC: 59% and 56.9%, CC: 0% and 9.2%; for rs2228262 (A>G) AA: 94.8% and 96.8%, AG: 5.1% and 3.1% in rejection and controls respectively. The allele and genotype frequency for the 3 described *THSB1* SNPs did not show any difference between the corneal graft immune rejection patients and controls. **Conclusion:** Asian Indian population evaluated for *THBS1* gene SNPs by ARMS PCR genotyping in Asian Indian population did not show any genetic association to immune rejection occurrence in our study.

Key words: Alleles, Amplification Refractory Mutation System-Polymerase Chain Reaction, corneal transplantation, genotype, rejection, single nucleotide polymorphism, thrombospondin-1

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Corneal grafting or cornea transplant is the only option for replacing the diseased corneal tissue in corneal eye disease (CED) with a healthy tissue, which has been received from an organ donor. CED is one of the most common causes of blindness and affects irrespective of age and sex. Keratoplasty is the treatment of choice for replacing the diseased corneal tissue in corneal diseases with donor corneal tissue. Corneal grafting surgery has good success despite the grafts not being HLA matched. This success of human leukocyte antigen (HLA) unmatched corneal grafts with minimal immunosuppression have been attributed to the immune privilege in the anterior chamber resulting in the prevention of allograft rejection.[1] Immune graft rejection remains one of the most importance concerns for corneal graft failure. Studies have shown that corneal graft survival for all indications is 90% at one year, which declines to 70% by 5 years. In patients with corneal graft rejection, the 5-year survival is 50% which declines to less than 35% at 10 years.[2,3] The loss of immune privilege of the cornea by the presence of stromal vascularization in all four corneal quadrants leads to an enhanced risk of rejection.[2] Rejection rates of 14% in avascular corneas have been described to increase to 32% in the presence of preoperative vascularized host corneal bed.[4]

The 2-year survival rate in low-risk grafts is about 90%,[5,6] with the 5 years and 10 years survival rates being 90% and 82% respectively.[7,8] Survival rate for 2 years in corneal grafting in high risk recipient beds is less than 50%.[5] Immune-mediated graft rejection carries a heightened threat of failure to the subsequent grafting with a reported cumulative increase in the risk of corneal graft rejection increased by a factor of 1.2 with every subsequent re-graft.[2] The corneal graft rejection rates rise to rates of 40%, 68%, and 80% after the first, second, and third re-grafts in these high-risk recipients' corneal bed.

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As corneal graft survival rates decrease with increased risk of graft rejection, it is imperative to explore strategies to evaluate the underlying molecular pathogenesis responsible for graft rejection and the factors for reducing the risk of corneal graft rejection.

The lack of vascularity in normal cornea prevents the direct access of the immune system to it while the lack of lymphatics limits the free transport of antigens and antigen processing cells (APCs) to T-cell-rich secondary lymphoid organs. The low expression of major histocompatibility (MHC) antigens (MHC-I and –II antigens) in all the layers of the cornea retards the onset of immunogenicity to foreign antigens. The dendritic cells (DCs) present in the central and peripheral cornea, exist in an immature, inactivated state, facilitating the immune privilege in normal healthy cornea. Several cell membrane-bound molecules expressed by the cornea that protect it from immune-mediated inflammation and enable apoptosis of immune effector cells include complement regulatory proteins (CRP), Fas ligand (FasL), MHC-Ib, and tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL).[9]

Soluble immunosuppressive factors abundant in the anterior chamber of the eye include the TGF-β, alpha melanocyte stimulating hormone (α-MSH), calcitonin gene-related peptide (CGRP), CRP, somatostatin (SOM), indoleamine dioxygenase (IDO), vaso-intestinal peptide (VIP), and macrophage migration inhibitory factor (MIF), which inhibit T cell and complement activation.[10]

The anterior chamber-associated immune deviation (ACAID) is of significant importance as a system of alloantigen-specific peripheral immune tolerance to antigens in the anterior chamber, which is capable of altering the systemic cytotoxic immune response.[11] ACAID promotes corneal graft survival by way of effecting the suppression of delayed-type hypersensitivity (DTH) response and maintaining the humoral immunity.[9]

Recent evidence strongly points to the matricellular glycoprotein, thrombospondin *THBS1*, as a key immunoregulatory factor. Thrombospondins are multidomain, calcium binding, extracellular glycoproteins, that maintain an anti-angiogenic environment in the eye and the human thrombospondin-1 glycoprotein gene (*THBS1*) is located on chromosome 15q15.[12] *THBS1* glycoprotein is expressed by the human corneal epithelial basement membrane, the corneal endothelium, posterior Descemet's membrane, the trabecular meshwork, lens epithelium, and blood vessels.[13] *THBS1* is known to be involved in the immune response of the anterior chamber of the eye by binding and activating latent TGF-β2 [Fig. 1].[14] TGF-β2 has an inhibitory effect on the activity of T lymphocytes, suppresses DCs maturation, and promotes the generation of phenotypically and functionally immature DCs, resident antigen-presenting cells (APCs) that are found in human corneal stroma in allograft rejections.[9,15] *THBS1* functions as a potent suppressor of immune rejection is by downregulating the capacity of APCs to induce allosensitization of T cells,[16] and by *THBS1* glycoprotein expression of the APCs impeding the APCs from embracing a phenotypically and functionally mature form. The discovery of this important function for *THBS1* glycoprotein in the transplant setting now directs future strategies to targeting upregulation of *THBS1* in APCs as an effective means to enhance allograft survival[16] and targeting *THBS1*-mediated TGF-β2 activation can enable new therapeutic approaches to corneal allograft rejections. The possibility of a genetic association to immune-mediated inflammation in corneal graft patients has been noted by an earlier study identifying three single nucleotide polymorphisms (SNPs) in the *THBS1* gene that have been postulated to influence the *THBS1* glycoprotein expression in the Caucasian population.[17] This prompted us to explore for the possibility of the same in Asian Indian population. This pilot study on *THBS1* gene polymorphisms in Asian Indians in corneal allograft rejection in penetrating keratoplasty patients has been undertaken to evaluate if these patients had a genetic predisposition to immune-mediated inflammation involved in corneal graft rejection.

Methods

This is a prospective case–control study of patients with optical penetrating keratoplasty and controls to evaluate for *THBS1* gene single nucleotide polymorphisms (SNPs). Institute ethics approval and informed consent were obtained from all recruited study subjects. The study conformed to the Declaration of Helsinki. Cases comprised of 58 patients of optical penetrating keratoplasty (45 patients of optical penetrating keratoplasty with history of immune graft rejection (group 1) and 13 patients with clear full thickness corneal grafts for a minimum of 3 years without any previous episodes of rejection (group 2) were recruited from the outpatient and follow-up keratoplasty clinic our tertiary care center between the period of January to December 2016. Patients with corneal graft failures due to definite nonimmunologic causes, such as primary graft failure, acquired infection, or recurrence of original disease and those not consenting for participation in the study were excluded. Sixty-five normal subjects between 15 years to 75 years (age and gender matched) were taken as controls (group 3). Data recorded for all recruited study subjects included demographic details relating to indication, details of corneal graft surgery, details of immune graft rejection, graft status, and visual acuity. Complete ocular examination including visual acuity, IOP, slit lamp biomicroscopy, graft status were done for all recruited patients.

Blood sample analysis for DNA from study recruits for genotyping for SNPs in *THBS1* gene in patients with high risk corneal recipient bed and controls to identify a genetic predisposition in the corneal graft rejection was the primary outcome that was evaluated.

Peripheral blood collected from each study subject was used for isolation of DNA. DNA was extracted by using Qiagen DNA isolation kit following manufacturer's instruction. Three *THBS1* gene SNPs (rs1478604, A>G; rs2228261, C>T; and rs2228262, A>G) that had been identified earlier[17] were analyzed by Amplification Refractory Mutation System-Polymerase Chain Reaction (ARMS PCR) method.

Tetra-ARMS PCR genotyping was designed to analyze the *THBS1* gene polymorphism as described below. Tetra-primer ARMS methodology [Fig. 2] utilizes two primer pairs to amplify the two different alleles of a given SNP in a single PCR reaction (Since these genotypes are single nucleotide polymorphisms, presence of either nucleotide, A or G/C or

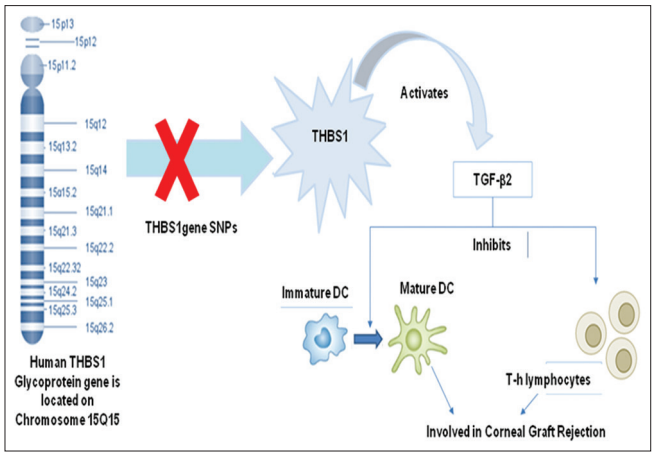

Figure 1: Simplified diagrammatic representation of role of *THBS1* (*THBS1* thrombospondin 1; SNP-single nucleotide polymorphism, TGF – Transforming growth factor; DC – dendritic cells; APCs – antigen presenting cells; Th – T helper)

T is normal. Different individuals in a population can have different genotypes and be normal). In this method, two allele-specific amplifications occur in opposite directions with two outer primers that amplify the region of the SNP and two inner allele-specific primers. In this method, the allele-specific primers have a mismatch at 3′ terminal base, but in addition they have a second deliberate mismatch at position 2 from the 3′ terminus. The inner primers have an average length of 28 bases in order to minimize the difference in stability of primers annealed to the target and nontarget alleles, ensuring that specificity results from differences in extension rate rather than hybridization rate. To achieve the required level of reliability and reproducibility, the tetra-primer ARMS-PCR technique requires an initial primer design analysis and optimization process. Four primers were designed for each SNP by Primer1 software [Fig. 3]. The common fragment length varied according to the SNPs and the primers used [Table 1]. PCR products were visualized under UV after running it in horizontal 3% agarose gel.

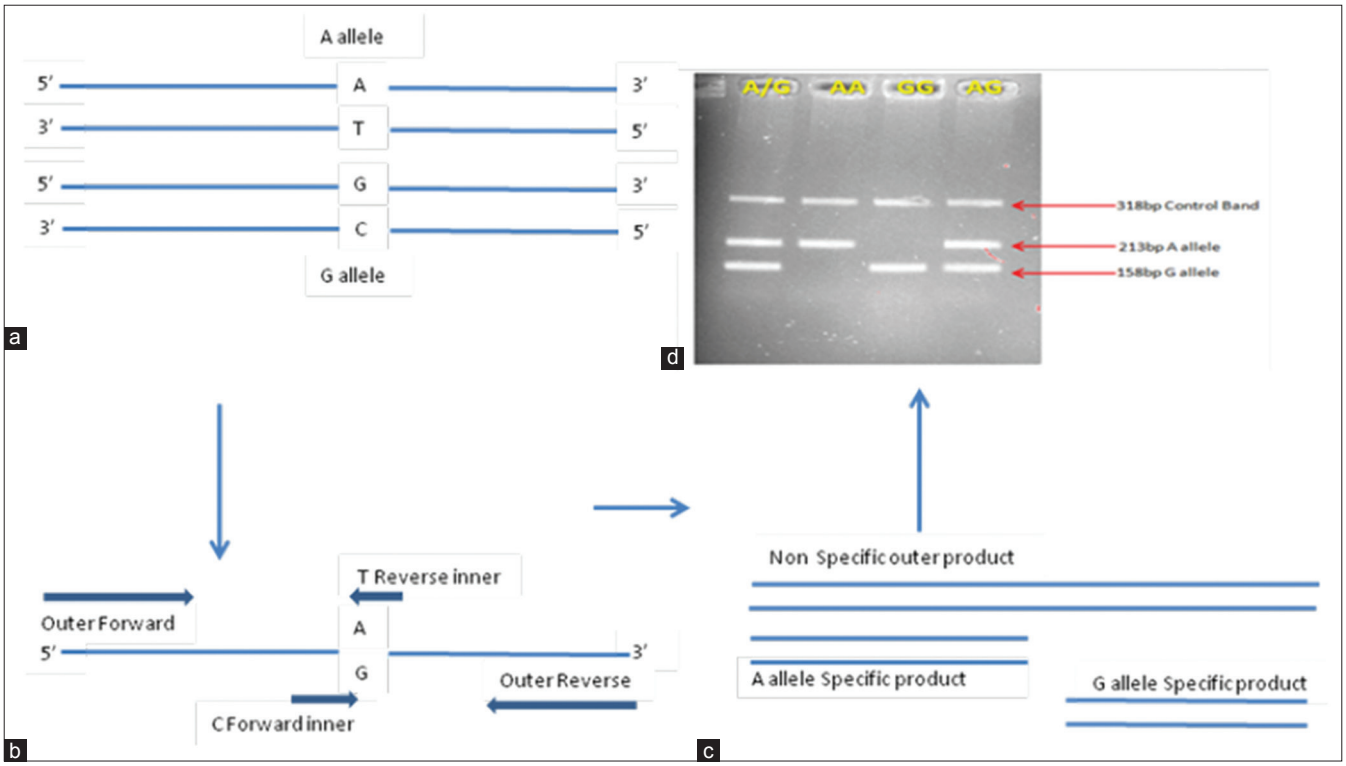

Figure 2: Diagrammatic representation of the ARMS–PCR assay for the A>G substitution SNP genotyping as an example. (a) both A and G allele with complementary base pair; (b) All four sets of primers and its binding positions; (c) respective amplified products according to the allele and with the non specific control non specific outer product and (d) corresponding gel picture

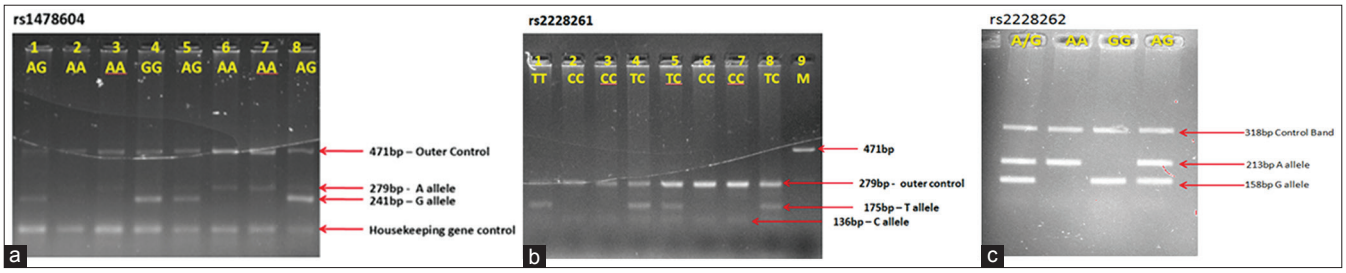

Figure 3: Genotyping for SNPs using 3 TSP-1 tagging SNPs rs1478604 A>G (a), rs2228261 C>T (b), rs2228262 A>G (c) by Tetra-ARMS PCR genotyping

Table 1: Tetra-primer Amplification Refractory Mutation System-Polymerase Chain Reaction primers for the 3 Single Nucleotide Polymorphisms

Single nucleotide polymorphism (SNP)	Tetra primers	Melting temperature	Product size
rs1478604	Forward inner primer (A allele):	79	Product size for A allele: 151
	121 AGCTGGCCTGCGAGTTCAGGGCTCCAGA 148		Product size for T allele: 137
	Reverse inner primer (T allele):	76	Product size of two outer primers: 230
	177 TCCGGAGTAGAGGTTGCTCCTGGAGAGGGA 148		
	Forward outer primer (5′ - 3′):	77	
	41 ATTGGCCGGAGGAATCCCCAGGAATGC 67		
	Reverse outer primer (5′ - 3′):	77	
	270 CGGGGGCGACTTACCTGTGTGTACCGGA 243		
rs2228261	Forward inner primer (C allele):	79	Product size for C allele: 160
	129 TCTGCAACTCTCCCAGCCCCCAGATGCAC 157		Product size for T allele: 139
	Reverse inner primer (T allele):	78	Product size of two outer primers: 243
	183 CGCGCTTCGCCTTCACAGGGTTTCACA 157		
	Forward outer primer (5′ - 3′):	78	
	45 AACAGGATGGTGGCTGGAGCCACTGGTCC 73		
	Reverse outer primer (5′ - 3′):	78	
	287 CAGATGCCAGGCAACCAGCTGGGCAG 262		
rs2228262	Forward inner primer (A allele):	74	Product size for A allele: 213
	238 GACACAGACCTGGATGGCTGGCCAAA 263		Product size for G allele: 158
	Reverse inner primer (G allele):	73	Product size of two outer primers: 318
	289 CATTGGCCACGCACACCAGGTTCTAAC 263		
	Forward outer primer (5′ - 3′):	73	
	132 CTGCAACAAGAACGCCAAGTGCAACTACC 160		
	Reverse outer primer (5′ - 3′):	73	
	449 GGAATTAGTGCCCCTCTCCCTTTGGGAGA 421		

All data were recorded on a predesigned proforma. Data was analyzed in three groups:

Group 1 – patients with corneal grafts with immune graft rejection, Group 2 – patients with corneal grafts without rejection, and Group 3 – Controls.

Statistical analysis

The quantitative data was compared using nonparametric Kruskal Wallis test for more than 2 groups. Qualitative data was compared using Chi square test. Spearman test was used for correlation analysis. *P*- value of <0.05 was considered significant.

Results

Demographic data of study subjects and controls

Our study evaluated 58 patients [33 male patients of mean age 32 ± 18.2 (range 2.5 to 71 years); 25 female patients of mean age 38.4 ± 25.1 (range 4 to 80 years)] who had undergone corneal grafting over a mean follow-up period of 73.5 ± 31.7 months (range 36 to 120 months). Of these, 45 patients of mean age 33.7 ± 21.5 years (range 2.5 to 74 years) [26 males (mean age 31.7 ± 19.1 years); 19 females (mean age 36.3 ± 24.7 years)] had experienced corneal graft rejection episodes (group 1).

The mean time of occurrence of immune graft rejection was at 39.3 ± 84.9 months (range 1.2 to 570 months) following corneal grafting surgery over a mean follow up of 79.73 ± 79.53 months (range 11 to 374 months); 33 patients had one episode of graft rejection at a mean time interval of 27.12 + 27.6 months (range 1.2 to 144) after the grafting while 11 eyes had two episodes at a mean time interval of 77.04 months (range 6 to 570 months) and 3 episodes of graft rejection in one patient at a mean time interval of 37.96 months.

Group 2 comprised of 13 patients of mean age of 38.5 ± 22 years (range 4 to 80 years) [7 males (mean age 33.14 ± 15.78; range 17–60 years); 6 females (mean age 44.8 ± 27.8; range 4–80 years)] who had undergone corneal grafting surgery and did not have any history of rejection over mean follow up period of 63.07 + 30.3 months (range 23–112 months). None of the recruited patients had vascularized host corneal beds. Sixty-five normal individuals of mean age 31.2 ± 11.3 years (range 16–74 years) [males - 46 (30.8 ± 11 years; range 16–61 years); females 19 (32.3 ± 12.2 years; range 22–74 years)] were recruited as control subjects (group 3).

Of the blood samples of the 45 patients with immune graft rejection following corneal grafting that were collected, DNA isolation could be performed in 38 samples. A total 116 blood samples were therefore analyzed for the three *THBS1* gene SNPs. The study group and the controls were population matched for age and gender. The demographic details, indications, time of surgery, time of occurrence of graft rejection are elaborated in Table 2 (corneal grafts with immune rejection) and Table 3 (corneal grafts without rejection). The details of the allele frequencies and genotype frequencies between the three groups are given in Tables 4 and 5 respectively. The allele frequencies were noted to be in Hardy Weinberg equilibrium.

Discussion

Documented mechanisms of the underlying ocular immune privilege of the cornea[18-23] include factors such as absence of blood and lymphatic vessels in the graft bed in low risk corneal grafts, an immunosuppressive ocular microenvironment due to regulatory molecules [TGF-β2, *THBS1*, a-MSH), VIP, CGRP], cortisol, and ACAID. Thrombospondins[24,25] help maintain an anti-angiogenic environment in the eye and *THBS1* is known

Table 2: Demographic characteristics of Patients with corneal grafts with immune graft rejection

No	Age/ sex	laterality	No of rejections	Time of Rejection after surgery (months)	no of grafts	Current graft status	Co-morbidity	Indication for corneal graft	fellow eye
1	10/M	OS	1	2	OU 2	Failed	Glaucoma	CHED	Failed graft Retained DM
2	6/F	OD	1	18	OU 1	Rejection reversed		CHED	Op PK - clear graft
3	26/F	OS	1	28	1	Rejection Reversed	Viral	Healed viral keratitis	Tectonic PK
4	51/F	OS	1	72	1	Failed	Glaucoma	LCO	WNL
5	40/M	OD	1	3	1	Rejection reversed	-	LCO	LCO
6	26/M	OS	2	30	1	Failed	-	CI scar	CI scar (KM)
7	60/M	OS	1	48	2	Clear	-	LCO	LCO
8	39/M	OS	1	13	1	Failed	-	LCO	WNL
9	33/F	OD	1	18	2	Clear	RIOFB removal ACIOL	Trauma CO	WNL
10	28/M	OD	1	36	1	Clear	-	CHED	CHED
11	71/M	OS	1	12	2 (rejection in 2nd graft)	Clear	-	LCO	Failed graft
12	73/F	OS	2	12	1	Clear	Glaucoma	FECD	FECD
13	22/F	OS	1	20	OD 2 OS 1	Clear	-	CHED	CHED
14	24/F	OD	1	35	OU 1	Clear	-	LCO	Viral endotheliitis
15	74/F	OS	OU 2	570	OU 1	Clear	-	LCO	Aphakia Op PK
16	15/M	OD	2	6	2	Failed	-	KC	keratoconus
17	9/M	OS	1	27	1	Clear	-	LCO	WNL
18	20/F	OS	1	4	1	Clear	-	Macular Dystrophy	Macular Dystrophy
19	57/M	OS	1	86	1	Failed	-	Viral K	CI scar
20	27/M	OD	1	35	1	Clear	-	Viral melt	WNL
21	30/F	OD	2	40	2	Failed	Glaucoma	Axenfeld Reiger's syndrome	Phthisis
22	15/F	OU	3	28.00	1	Clear	Glaucoma	KC	KC+C3R
23	18.5/M	OD	2	17	3	Clear	-	CHED	CHED
24	33/M	OD	1	19	1	Clear	-	Macular Dystropy	Macular Dystrophy
25	16/M	OS	2	65	1	Clear	-	WNL	LCO
26	14/F	OD	1	21	1	Clear	-	WNL	LCO (viral)
27	26/M	OD	1	6	1	Failed	-	CHED	CHED
28	2.5/M	OD	1	13	2	Failed	-	Phthisis	LCO (KM)
29	62/F	OD	1	27	1	Failed	-	CO	LCO
30	41/M	OS	2	24	1	Failed	-	LCO	LCO
31	13/M	OD	1	16	1	Failed	-	WNL	LCO
32	17/F	OS	1	10	1	Clear	-	WNL	LCO (IK)
33	60/M	OD	1	24	1	Failed	-	LCO	LCO (viral)
34	68/F	OS	2	47	1	Clear	Glaucoma	LCO	LCO
35	6/F	OD	1	1.2	2	Failed	-	WNL	CI scar
36	30/M	OS	1	144	1	Clear	OS High myopia + RD: Op VR	CHED	CHED
37	18/M	OS	1	18	1	Clear	Steroid induced glaucoma + op trab pseudophakia	KC + VKC	KC + VKC + C3R
38	70/F	OS	2	6	2	Failed	-	Failed graft	Aphakia+PK
39	46/M	OS	1	12	1	Clear	-		

Contd...

Table 2: Contd...

No	Age/ sex	laterality	No of rejections	Time of Rejection after surgery (months)	no of grafts	Current graft status	Co-morbidity	Indication for corneal graft	fellow eye
40	23/M	OS	2	31	1	Clear	-	Post LASIK Keratitis LCO	WNL
41	25/M	OS	1	32.00	1	Clear	-	KC	KC
42	65/F	OS	1	6	1	Failed	-		
43	21/M	OD	1	6.00	1	Failed	-		
44	15/F	OD	1	60	1	Failed	-		
45	71/M	OS	1	24	1	Failed	-		

WNL: Within normal limits; CHED: Congenital hereditary endothelial dystrophy; KC: Keratoconus; VKC: Vernal keratoconjunctivitis; PK: Penetrating keratoplasty; LCo: Leucomatous corneal opacity; C3R: Collagen crosslinking; IK: Infectious keratitis; RD: Retinal detachment; VR: Vitreoretinal surgery; KM: Keratomalacia; CI: Corneo-iridic; FECD: Fuch's endothelial dystrophy; Op: Optical

Table 3: Demographic characteristics of patients with clear corneal grafts without immune graft rejection

No	Age	Sex	laterality	fellow eye	diagnosis	Postop (months)	UCVA	BCVA
1	27	F	OD	WNL	LCO (IK)	48	3/60.	6/18
2	30	M	OS	WNL	Aphakia	48	2/60	6/36
3	17	M	OS	KC	KC	48	6/18	6/9
4	4	F	OD	LCO (IK)	WNL	48	4/60	6/36
5	60	M	OD	LCO (IK)	WNL	48	0.3/60	6/60
6	61	F	OS	op trab+BK	IMSC	70	6/24	6/9
7	49	M	OD	Viral K	Op PK + pseudophakia (mac dystrophy)	108	6/24	NA
8	23	M	OS	VKC+KC	Op PK	36	6/36	6/6
9	35	F	OD	Macular dystrophy	Op PK	120	6/36	6/18
10	62	F	OD	Adherent leucoma	Op PK+aphakia	120	6/24	6/12
11	32	M	OS	WNL	Op PK+pseudophakia	58	6/36	6/18
12	21	M	OD	WNL	Op PK (LCO)	96	6/18	6/9
13	80	F	OD	Viral K	Op PK+aphakia	108	6/24	6/18

WNL: Within normal limits; CHED: Congenital hereditary endothelial dystrophy; KC: Keratoconus; VKC: Vernal keratoconjunctivitis; PK: Penetrating keratoplasty; LCo: Leucomatous corneal opacity; C3R: Collagen crosslinking; IK: Infectious keratitis; RD: Retinal detachment; VR: Vitreoretinal surgery; KM: Keratomalacia; CI: Corneo-iridic; FECD: Fuch's endothelial dystrophy, trab: Trabeculectomy; Op: Optical; NA: Not available; K: Keratitis; BK: Bullous keratopathy

Table 4: Details of allele frequencies in the study groups

	Rejection group	Clear graft	Control group
rs1478604(A>G)			
f(A)	69.7%	46.1%	72.6%
f(G)	30.2%	53.8%	27.3%
rs2228261(C>T)			
f(T)	70.2%	76.9%	62.3%
f(C)	29.7%	23%	37.6%
rs2228262(A>G)			
f(A)	97.4%	96.1%	98.4%
f(G)	2.5%	3.8%	1.5%

to be involved in the immune response of the anterior chamber of the eye, by binding and activating latent TGF-β2,[14] thereby facilitating peripheral and systemic tolerance in allograft rejections,[17,26] and thwarting the proangiogenic activity of VEGF.[27]

The potential anti-lymphangiogenic therapeutic effects of *THBS1* glycoprotein suggest that targeting *THBS1*-mediated TGF-2 activation can enable new therapeutic approaches for treatment of corneal neovascularization in high risk corneal graft patients and increase corneal graft survival rates.[9,17] Graft survival can perhaps also be promoted by targeted upregulation of *THBS1* glycoprotein in the antigen presenting cells. The presence of *THBS1* gene SNPs has been shown to interfere with the corneal immune privilege thereby providing a genetic predisposition to immune graft rejection.[17] Genotype frequency which may also be referred to as genomic profiling can help to predict a person's genetic predisposition to a particular disease or event. Hence this current study of *THBS1* gene polymorphisms in Asian Indians in corneal allograft rejection patients has been undertaken to evaluate if the eyes of Asian Indians has a genetic predisposition to immune-mediated inflammation as noted by the earlier study in Caucasian population.[17] This is a study of patients with corneal grafts to evaluate for *THBS1* gene SNPs in 38 of the 45 patients of corneal grafting with history of immune graft rejection (group 1) and 13 patients with clear grafts without any previous episodes of rejection (group 2) and 65 normal subjects (group 3). The frequencies of allele for rs1478604 (A>G) A was found to be 69.7% and 72.6%; for G was 30.2% and 27.3% in the corneal graft with immune rejection patients and the

Table 5: Details of Genotype frequencies for TSP-1 SNPs in the study groups

	Rejection group	No rejection group	Control group	*P**
rs1478604(A>G)				
f(AA)	57.8%	30.7%	59.3%	0.299
f(AG)	23.6%	30.7%	26.5%	
f(GG)	18.4%	38.4%	14%	
rs2228261(C>T)				
f(TT)	39.4%	58.3%	33.8%	0.165
f(TC)	60.5%	33.3%	59.6%	
f(CC)	0	8.3%	6.45%	
rs2228262(A>G)				
f(AA)	94.8%	92.3%	96.8%	0.535
f(AG)	5.1%	7.6%	3.1%	
f(GG)	0	0	0	

*Fisher Exact Test

control population respectively (not statistically significant). The frequencies of allele for rs2228261(C>T) T was found to be 70.2% and 62.3%; for C was 29.7% and 37.6% in the corneal graft with immune rejection patients and the control population respectively not statistically significant. This implies that the frequency of occurrence of SNP for thymine and cytosine was similar in both the immune rejection patients and controls. The frequencies of allele for rs2228262 (A>G) A were found to be 97.4% and 98.4%; for G was 2.5% and 1.5% in the corneal graft with immune rejection patients and the control population respectively (not statistically significant). The occurrence of frequency of SNP for adenine and guanosine was also found to be similar in both the immune graft rejection patients and controls.

Similarly on looking at the frequencies of genotype for rs1478604 (A>G) AA (homozygosity for adenine) was found to be 57.8% and 59.3%; for AG (heterozygosity for adenine) 23.6% and 26.5%; for GG (homozygosity for cytosine) was 18.4% and 14% in the corneal graft with immune rejection patients and the control population respectively (not statistically significant), which implies that there was not much difference of their occurrences in both the groups. The frequencies of genotype frequencies for rs2228261(C>T) TT (heterozygosity for thymine) were found to be 40.5% and 33.8%; for TC (heterozygosity) 59% and 56.9%; for CC (homozygosity for cytosine) was 0% and 9.2% in the corneal graft with immune rejection patients and the control population respectively (not statistically significant), which implies that there was again not much difference in the genotype frequency occurrences between the two groups. The frequencies of genotype frequencies for rs2228262 (A>G) AA were found to be 94.8% and 96.8%; for AG 5.1% and 3.1% in the corneal graft with immune rejection patients and the control population respectively (not statistically significant).

From our results, there seems to be no significant difference in the genotype frequencies of the three markers for *THBS1* gene SNPs between the rejection and the control group. The allele frequency between the study groups also does not show a significant difference. An earlier study by Winton *et al*.,[17] evaluating the role of SNPs of *THBS1* gene in Caucasian population on the risk of corneal allograft rejection analyzed 378 corneal graft patients with risk factors for allograft rejection and found that THBS-1 rs1478604A SNP was associated significantly with an increased risk of corneal allograft rejection (odds ratio [OR], 1.58; 95% confidence interval [CI], 1.02–2.45; *P* ¼ 0.04). Their study also showed a trend toward the rs1478604, rs2228261, rs2228262 ACA haplotype increasing risk of rejection. This led them to suggest that *THBS1* rs1478604 AA homozygotes may be at increased risk of immune rejection following corneal grafting surgery especially if they harbor the ACA haplotype. However, our data does not seem to predict the association of any allele with rejection of corneal graft. A larger sample size study can perhaps re-evaluate for this association.

Conclusion

In conclusion, genetic predisposition for occurrence of immune corneal allograft rejection in form of the reported three SNPs in *THBS1* glycoprotein is not noted in our Asian Indian population.

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Conflicts of interest

There are no conflicts of interest.

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