VEGFA Promoter Polymorphisms rs699947 and rs35569394 Are Associated With the Risk of Anterior Cruciate Ligament Ruptures Among Indian Athletes

A Cross-sectional Study

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Background: Associations of genetic variants within certain fibril-forming genes have previously been observed with anterior cruciate ligament (ACL) injuries. Evidence suggests a significant role of angiogenesis-associated cytokines in remodeling the ligament fibril matrix after mechanical loading and maintaining structural and functional integrity of the ligament. Functional polymorphisms within the vascular endothelial growth factor A (VEGFA) gene have emerged as plausible candidates owing to their role in the regulation of angiogenic responses.

Hypothesis: VEGFA promoter polymorphisms rs699947 and rs35569394 are associated with ACL injury risk among athletes.

Study Design: Cross-sectional study; Level of evidence, 3.

Methods: A total of 90 Indian athletes with radiologically confirmed or surgically proven isolated ACL tears and 76 matched-control athletes were selected for the present cross-sectional genetic association study. Oral mouthwash samples were collected from all the case and control athletes and genotyped for VEGFA rs699947 and rs35569394 using the polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) method.

Results: The A allele (rs699947) was significantly overrepresented in the ACL group (C vs A allele: odds ratio [OR], 1.68 [95% CI, 1.08-2.60]; $P = .021$) (CC vs CA + AA: OR, 2.69 [95% CI, 1.37-5.26]; $P = .004$). There was a greater frequency of the AA genotype in the ACL group in comparison with the control group (OR, 3.38 [95% CI, 1.23-9.28]; $P = .016$) when only male athletes were compared. Likewise, there was a greater frequency of the I allele (rs35569394) in the ACL group (D vs I allele: OR, 1.64 [95% CI, 1.06-2.55]; $P = .025$) (DD vs ID + II: OR, 2.61 [95% CI, 1.31-5.21]; $P = .006$). The A-I haplotype was overrepresented in the ACL group compared with the control group (OR, 1.68 [95% CI, 1.08-2.60]; $\chi^2 = 5.320$; $P = .021$), and both the polymorphisms were found to be in complete linkage disequilibrium ($r^2 = 0.929$; logarithm of the odds score = 63.74; D′ = 1.0). Female athletes did not show any difference in genotype or allele frequency.

Conclusion: This is the first study to investigate the association of VEGFA promoter polymorphisms in ACL tears among Indian athletes. Increased frequencies of the A allele (rs699947) and I allele (rs35569394) were observed in the ACL group. These results suggest that sequence variants in the VEGF gene are associated with ACL injury risk among athletes. Further research with long-term follow-ups measuring VEGF expression levels during recovery is warranted to establish its role in ACL injuries and healing.

Keywords: ACL; athletes; VEGFA; promoter polymorphisms; genotyping; haplotype

Anterior cruciate ligament (ACL) injuries account for almost 50% of all knee injuries occurring in sports.[11,25] Trauma to the ACL compromises translational and rotational stability of the knee in the anterior joint space in all

cardinal planes[16] and often results in chondral lesions, followed by posttraumatic osteoarthritis.[13]

Extracellular matrix remodeling[32] via the increased expression of several proangiogenic cytokines[17,18] has been reported in tendons in response to mechanical stimuli. Angiogenesis-associated signaling pathways have been investigated in relation to certain orthopaedic conditions as well.[35] Recently, genomic sequence variants among genes modulating the angiogenesis-associated signaling pathway have been associated with the susceptibility of ACL injuries.[17-19] Vascular endothelial growth factor (VEGF), encoded by the VEGF gene, is an essential component in the regulation of angiogenesis-associated signaling pathways.[8] The VEGF gene is located on chromosome 6 (6p21.1), consists of 8 exons,[4] and has at least 30 reported single nucleotide polymorphisms (SNPs) within 5 isoforms: VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor.[34] The VEGF-A isoform has shown the highest angiogenic potency,[19] and one of its promoter SNPs, VEGFA rs699947 (-2578 C>A; 6:43768652), has been linked to a number of pathophysiological conditions, including coronary artery disease,[33] cancer,[18] rheumatoid arthritis,[10] and certain tendinopathies.[14,18,20]

VEGFA rs35569394 (18bp I/D; 6:43736418) is an 18-bp insertion/deletion polymorphism located at the -2549 position in the promoter region of the VEGF gene and has been found to be associated with increased angiogenesis in several conditions such as diabetic nephropathy,[1] diabetic retinopathy,[5] and certain types of cancers.[11] Although a potent angiogenic site, the role of rs35569394 (-2549 18bp I/D) has not been investigated in musculoskeletal soft tissue injuries.

Understanding the role of DNA variations in the risk of ACL injuries is paramount for the personalized treatment of athletes to aid healing and the timely return to sports. India, globally the second most populous nation, is also the most diverse genetically.[22] Thus far, limited data are available on the genetic risk factors for an ACL tear in the Indian population, as most of the studies have focused on White populations. Therefore, this study aimed to identify any association between VEGFA SNPs (rs699947 and rs35569394) and ACL injury susceptibility among an Indian athletic population. To the best of our knowledge, this is the first study that reports the association of the rs35569394 polymorphism with ACL injuries.

METHODS

Participants

A total of 90 athletic patients (75 male and 15 female) with either radiologically confirmed or surgically proven ACL tears participated as cases in the study. Patients were included if they met the following criteria: (1) participation in any contact or noncontact sports, (2) age between 18 and 35 years, (3) isolated ACL injuries with no multiligament or meniscal injury or signs of osteoarthritis in the knee, (4) an ACL tear during training or competition, and (5) not suffering from any chronic disease. In addition, 76 unrelated, healthy, physically active athletes (59 male and 17 female) matched to ACL athletic patients in terms of age, sex, nature of sports played, and training regimen, with no self-reported history of knee ligament/tendon injuries, were recruited as controls. All the athletes were recruited from various sports injury clinics, orthopaedic rehabilitation centers, and prominent sports training academies in India from September 2018 to May 2019. After providing informed consent, every case and control athlete underwent a thorough clinical evaluation and completed a questionnaire consisting of general details (name, age, sex, state of domicile) as well as details about their sporting career, mechanism of injury and treatment, and personal and family history of such trauma. Athletes with an ACL injury were stratified according to mechanism of injury into contact (n = 28) versus noncontact (n = 62) groups in accordance with the classification system of the American Orthopaedic Society for Sports Medicine.[15] This study was approved by an institutional human ethics committee and was conducted in accordance with the guidelines of the World Medical Association Declaration of Helsinki (2013).

DNA Extraction

To extract genomic DNA, oral mouthwash samples were obtained from athletes in a sterile container containing 2 mL sodium chloride–tris–EDTA buffer (100 mM NaCl; 10 mM Tris-Cl; pH 8.0; 1 mM EDTA). Genomic DNA was extracted from the oral epithelial cells using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's protocol. The eluted DNA was quantified using NanoPhotometer P330 (Implen), electrophoresed in 1× TAE 0.8% agarose gel, and visualized by ethidium bromide staining.

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VEGFA Genotyping

Standard genotyping protocols including polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis were employed. The manufacturer's instructions were followed for all the reactions, with minor modifications. Details about primers and reaction conditions are provided in Appendix Table A1. For VEGFA rs699947 screening, PCR amplicons were digested with FastDigest *Bgl*II (Thermo Fisher Scientific). The PCR amplicons of rs35569394 and digested products of rs699947 were electrophoresed in 2.5% agarose gel and visualized by ethidium bromide staining. Figure 1A shows a schematic view of a VEGF gene fragment targeted for PCR-RFLP. The lengths of PCR- and PCR-RFLP digested products are shown in Figure 1B and 1C. Genotypes were determined based on the length of the DNA fragments calculated by electrophoresis of the DNA ladder (Figure 1, B and C). Additionally, PCR products of 9 random samples were sequenced by the BigDye Terminator Cycle Sequencing Kit (Thermo Fisher Scientific) to further confirm the RFLP results (Figure 1, B and D). Multiple attempts to sequence the 476-bp amplicon of the rs699947 CA genotype (heterozygous) were unsuccessful because of the significant association of the ID-CA genotype (multiple sequence alignment) (Figure 1E), which perhaps hampered capillary electrophoresis of the BigDye Terminator–labeled DNA samples in the DNA sequencer. Thus, amplicons of 214 bp and 196 bp were eluted separately and sequenced to confirm the CA genotype of randomly selected samples (data not shown).

Statistical Analysis

Association analysis was performed using SPSS 22.0 software (IBM) and GraphPad InStat Version 3 (GraphPad Software). Continuous variables were reported as the mean ± SD. Data normality was assessed using the Shapiro-Wilk test, and the independent-samples t test or Mann-Whitney U test was performed to investigate any difference between the control, ACL, and noncontact ACL groups accordingly. The genotype and allele frequencies and other between-group categorical comparisons were performed using the chi-square test or Fisher exact test. The association analysis in the present study was performed in 2 ways: (1) general association with a 2×3 contingency table and (2) association analysis of 3 different genetic models: additive model, dominant inheritance model for minor alleles, and recessive inheritance model for minor alleles. Allele-based odds ratios (ORs) and 95% CIs were calculated for the ACL injury risk. Haplotypes were calculated from genotyped data, and haplotypes and linkage disequilibrium (LD) were calculated using Haploview 4.2 software (Broad Institute, Cambridge, Massachusetts). The Hardy-Weinberg equilibrium was determined using the online calculator from the Online Encyclopedia for Genetic Epidemiology Studies (https://www.oege.org/software).[23] P values <.05 were considered statistically significant.

RESULTS

Participant Characteristics

Because sampling was conducted previously and several extracted DNA samples failed in PCR amplification afterward, the remaining samples were genotyped for polymorphisms. Failure of PCR reactions when oral mouthwash DNA is used as a template is a common occurrence. Also, some participants were initially included but later excluded from analysis, as they did not meet all the inclusion criteria. Thus, overall, 76 control athletes and 90 ACL athletic patients were analyzed for 2 promoter polymorphisms within VEGFA: rs699947 and rs35569394. We performed a sex-based comparison for continuous characteristics. Demographic details of these participants are presented in Table 1.

In the present cross-sectional study, the athletes in the control and ACL groups were matched for age, height, weight, and body mass index (BMI) (Table 1). However, the ACL group was significantly heavier ($P < .01$) and also had a significantly higher BMI than the control group ($P < .01$) (Table 1). The athletes belonged to different sporting disciplines, and most ACL injuries occurred in contact games such as wrestling, judo, and throwing events in track and field. Also, patients in the ACL group were contacted during the rehabilitation phase at clinics and training facilities; complete ACL rehabilitation took place between 6 and 9 months[21] after surgical reconstruction/nonoperative management, and we required patients to avoid any kind of vigorous activity, as it might lead to increases in weight and BMI in the ACL group compared with the control group. Further, most of the patients in the ACL group underwent delayed ACL reconstructive surgery, thus adding to inactivity and subsequent increases in weight and BMI. No ACL cases or controls reported any injury to other knee joint structures or any chronic illness. Hardy-Weinberg equilibrium principles were met for the genotype distribution in all the groups (Table 2).

Genotype and Allele Frequency Analysis

The distribution of genotype and allele frequencies of both the polymorphisms is presented in Tables 2 and 3, respectively (Figure 2). Male athletes in the control and ACL groups showed a significant difference in the genotype and allele frequency distribution, as presented in Appendix Tables A2 and A3. Because the sample size of female athletes was smaller across all the groups, no meaningful conclusion could be drawn toward a phenotype-by-genotype association analysis of either polymorphism between the control, ACL, and noncontact ACL groups.

Haplotype Analysis and LD

Results of haplotype analysis and LD between VEGFA polymorphisms (rs699947 and rs35569394) are presented in Table 4. There were 3 common haplotypes for VEGFA rs699947 C/A and VEGFA rs35569394 18-bp I/D (C-D, C-I, A-I) that were calculated for 2 polymorphisms, and the C-D haplotype was significantly overrepresented in the control group (OR, 1.64 [95% CI, 1.06-2.55]; $\chi^2 = 5.024$; $P = .025$)
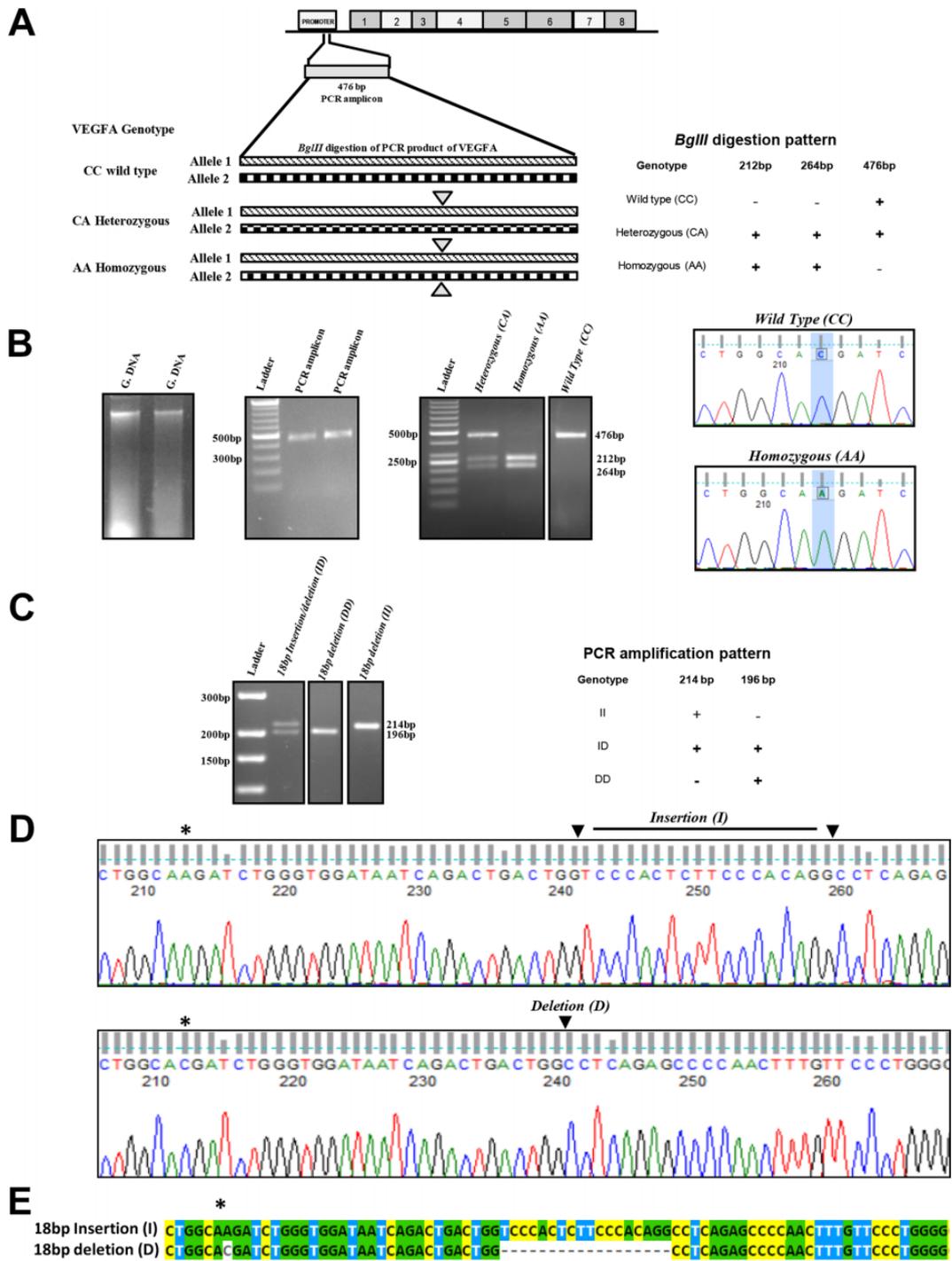
Figure 1. Polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) and sequencing of the vascular endothelial growth factor A (VEGFA) promoter. (A) Schematic view of a VEGF gene fragment targeted by PCR, followed by restriction digestion with *Bgl*II for PCR-RFLP assay. (B) Agarose gel electrophoresis of genomic DNA, PCR amplification of rs699947, *Bgl*II restriction digestion, and Sanger sequencing electropherogram for rs699947 wild-type and homozygous samples. (C) Agarose gel electrophoresis and PCR amplification pattern for rs35569394. (D) Sanger sequencing electropherogram showing the association of the A, I, C, and D alleles. Asterisk indicates the C/A allele, and block arrows show the I/D genotype location. (E) Multiple sequence alignment showing the I/D allele and association with the C/A allele.

TABLE 1
Characteristics of Study Participants[a]

	Control	ACL	P Value: Control vs ACL	Noncontact ACL	P Value: Control vs Noncontact ACL
All participants, n (%)	76 (100.0)	90 (100.0)		62 (100.0)	
Age, y	24.2 ± 4.1	26.6 ± 6.2	**.02**[b]	26.4 ± 6.3	.08
Height, cm	169.4 ± 9.4	171.2 ± 7.2	.18	171.2 ± 7.3	.24
Weight, kg	63.9 ± 10.0	74.1 ± 11.3	**<.01**[c]	73.6 ± 11.9	**<.01**[c]
BMI, kg/m^2	22.2 ± 2.2	25.2 ± 3.1	**<.01**[b]	25.0 ± 3.4	**<.01**[b]
Female participants, n (%)	17 (22.4)	15 (16.7)		7 (11.3)	
Age, y	22.6 ± 1.8	23.4 ± 2.9	.34	22.8 ± 2.2	.75
Height, cm	159.6 ± 6.4	162.8 ± 6.6	.18	159.6 ± 5.7	.98
Weight, kg	53.4 ± 5.8	65.6 ± 12.2	**.01**[c]	63.7 ± 16.3	**.03**[b]
BMI, kg/m^2	20.9 ± 1.8	24.7 ± 4.6	**<.01**[b]	25.0 ± 6.5	**.03**[b]
Male participants, n (%)	59 (77.6)	75 (83.3)		55 (88.7)	
Age, y	24.7 ± 4.5	27.2 ± 6.5	**.03**[b]	26.8 ± 6.6	.13
Height, cm	172.3 ± 8.1	172.8 ± 6.1	.29	172.6 ± 6.0	.37
Weight, kg	66.9 ± 8.8	75.7 ± 10.4	**<.01**[c]	74.8 ± 10.7	**<.01**[c]
BMI, kg/m^2	22.5 ± 2.1	25.3 ± 2.7	**<.01**[c]	25.0 ± 2.8	**<.01**[b]

[a]Data are reported as mean ± SD unless otherwise indicated. Data sets among groups were normally distributed except for age and BMI ($P < .05$). Bolded P values indicate statistical significance ($P < .05$). ACL, anterior cruciate ligament; BMI, body mass index.
[b]Mann-Whitney U test.
[c]Student t test.

TABLE 2
2×3 Phenotype-by-Genotype Association Analyses of VEGFA Polymorphisms With ACL Injury Risk[a]

	Control (n = 76)	ACL (n = 90)	Control vs ACL		Noncontact ACL (n = 62)	Control vs Noncontact ACL	
			P Value	OR (95% CI)		P Value	OR (95% CI)
VEGFA rs699947							
CC	33 (43)	20 (22)	>.99	>0.99	14 (23)	>.99	>0.99
CA	30 (40)	51 (57)	**.004**	2.80 (1.37-5.74)	35 (56)	**.011**	2.75 (1.24-6.08)
AA	13 (17)	19 (21)	.052	2.41 (0.98-5.92)	13 (21)	.087	2.36 (0.88-6.35)
χ^2 value (P value)			8.64 (**.013**)			6.71 (**.035**)	
Hardy-Weinberg equilibrium	0.18	0.21			0.30		
VEGFA rs35569394							
DD	30 (40)	18 (20)	>.99	>0.99	12 (19)	>.99	>0.99
ID	33 (43)	52 (58)	**.009**	2.63 (1.27-5.45)	36 (58)	**.015**	2.73 (1.20-6.19)
II	13 (17)	20 (22)	**.041**	2.56 (1.03-6.37)	14 (23)	.051	2.69 (0.98-7.38)
χ^2 value (P value)			7.61 (**.022**)			6.53 (**.038**)	
Hardy-Weinberg equilibrium	0.45	0.14			0.20		

[a]Data are reported as n (%) unless otherwise indicated. P values are unadjusted. Bolded P values indicate statistical significance ($P < .05$). ACL, anterior cruciate ligament; OR, odds ratio; VEGFA, vascular endothelial growth factor A.

compared with the ACL group, and the A-I haplotype was found overrepresented in the ACL group compared with the control group (OR, 1.68 [95% CI, 1.08-2.60]; $\chi^2 = 5.320$; $P = .021$). The r^2 value was 0.929, which is considered to be high LD (range, 0.70-0.98),[9] with a logarithm of the odds score of 63.74 and a D' of 1.0 (polymorphisms have the strongest LD) (Appendix Figure A1).

DISCUSSION

Angiogenesis-associated signaling is considered a key modulator of fibroblast cytoskeletal matrix remodeling after cyclic mechanical loading during complex sporting maneuvers, with VEGFA having the highest angiogenic potency.[19] Conflicting observations for genomic sequence variants within the VEGF gene have previously been reported for the ACL injury risk[14,19,20] and tendinopathies[18,24] in multiple White populations. Thus, we hypothesized that polymorphisms within the VEGFA promoter region (rs699947 and rs35569394) could be positively associated with the ACL rupture risk in the Indian athletic population.

VEGFA (rs699947 and rs35569394) Genotype and Allele Association

The results of our study revealed that there was a significantly greater frequency of the VEGFA rs699947 (-2578

TABLE 3
Analysis of Selected Polymorphisms Based on 3 Genetic Models for ACL Injury Risk[a]

	Control	ACL	Control vs ACL		Noncontact ACL	Control vs Noncontact ACL	
			χ^2 Value (*P* Value)	OR (95% CI)		χ^2 Value (*P* Value)	OR (95% CI)
VEGFA rs699947							
Additive			5.32 (**.021**)	1.68 (1.08-2.60)		4.27 (**.039**)	1.66 (1.02-2.69)
C	96 (63)	91 (51)			63 (51)		
A	56 (37)	89 (49)			61 (49)		
Dominant (CC vs CA + AA)			8.52 (**.004**)	2.69 (1.37-5.26)		6.60 (**.010**)	2.63 (1.24-5.56)
C	33 (43)	20 (22)			14 (23)		
A	43 (57)	70 (78)			48 (77)		
Recessive (CC + CA vs AA)			0.43 (.514)	1.30 (0.59-2.84)		0.33 (.564)	1.28 (0.55-3.02)
C	63 (83)	71 (79)			49 (79)		
A	13 (17)	19 (21)			13 (21)		
VEGFA rs35569394							
Additive			5.02 (**.025**)	1.64 (1.06-2.55)		4.53 (**.033**)	1.68 (1.04-2.72)
D	93 (61)	88 (49)			60 (48)		
I	59 (39)	92 (51)			64 (52)		
Dominant (DD vs ID + II)			7.60 (**.006**)	2.61 (1.31-5.21)		6.53 (**.011**)	2.71 (1.25-5.93)
D	30 (39)	18 (20)			12 (19)		
I	46 (61)	72 (80)			50 (81)		
Recessive (DD + ID vs II)			0.68 (.410)	1.38 (0.64-3.01)		0.65 (.420)	1.41 (0.61-3.28)
D	63 (83)	70 (78)			48 (77)		
I	13 (17)	20 (22)			14 (23)		

[a]Data are reported as n (%) unless otherwise indicated. *P* values are unadjusted. Bolded *P* values indicate statistical significance ($P < .05$). ACL, anterior cruciate ligament; OR, odds ratio; VEGFA, vascular endothelial growth factor A.

C>A) CA genotype in the ACL group and noncontact ACL subgroup, with a 2.80- and 2.75-fold more risk of ACL rupture compared with the asymptomatic control group, respectively (Table 2 and Figure 2). The C allele has been reported to enhance VEGF expression,[19] which might assist in matrix remodeling after cyclic loading, thus restoring the structural integrity and functional properties of the ligament.[3] Rahim et al[19] reported that the frequency of the C allele was notably higher in the ACL rupture group, with the C allele thus being the risk allele. However, our results demonstrated that the frequency of the A allele was high in the ACL group, thus opposing the findings of Rahim et al. Our study concentrated specifically on Indian athletes, and the observation of Rahim et al is based on an African population, thus racial/genetic variations between Africans and Indians may be a reason for the difference in findings.

Additionally, the dominant model for minor alleles (CC vs CA + AA) again suggested the A allele was overrepresented in the ACL and noncontact ACL groups, with a 2.69- and 2.63-fold more risk of sustaining ACL injuries than the asymptomatic control group (Table 3). We also noted an independent association of genotype and allele frequency of rs699947 with ACL injury susceptibility among male patients, and the A allele was overrepresented in the ACL and noncontact ACL groups, with a 3.01- and 3.23-fold more risk of ACL injuries, respectively (Appendix Table A2).

VEGFA rs699947 was in complete LD with another promoter polymorphism VEGFA rs35569394.[4,12] A patient having the A allele at the -2578 position

(rs699947) also had an 18-bp insertion at the −2549 position in the promoter region of the VEGF gene, while a patient with the C allele had an 18-bp deletion at the same location.[12] In our study, the frequencies of the ID and II genotypes and the I allele (rs35569394) were significantly higher in the ACL group, with a 1.64-fold more risk of ACL rupture than the control group, and because a noncontact mechanism is an important subcategory for the internal locus of causality of ACL ruptures, it is essential to report the OR associated with this category as well (Table 2 and Figure 2). Kapahi et al[12] suggested that the D allele of rs35569394 leads to enhanced transcriptional activity of the VEGF gene, resulting in an increased angiogenic response. Because the ACL is a hypovascular structure,[26,31] Cook and Docking[6] suggested that any significant increase in angiogenesis might lead to increased healing responses but compromised ACL strength. Also, Takayama et al[29] found that a low dose of VEGF (25%) was most effective in promoting healing and restoring biomechanical strength of ACL-reconstructed knees compared with a high dose of VEGF (100%), and blocking angiogenesis with anti-VEGF sFLT1 (soluble fms-like tyrosine kinase 1) significantly reduced the biomechanical strength and healing capacity of the ACL in a rat model. Results from our study suggest that optimally enhanced VEGF activity might be protective against musculoskeletal soft tissue injuries such as ACL ruptures. Ethnic differences in the genomic distribution may explain the variability in the findings of phenotype-by-genotype association studies on multiple populations.
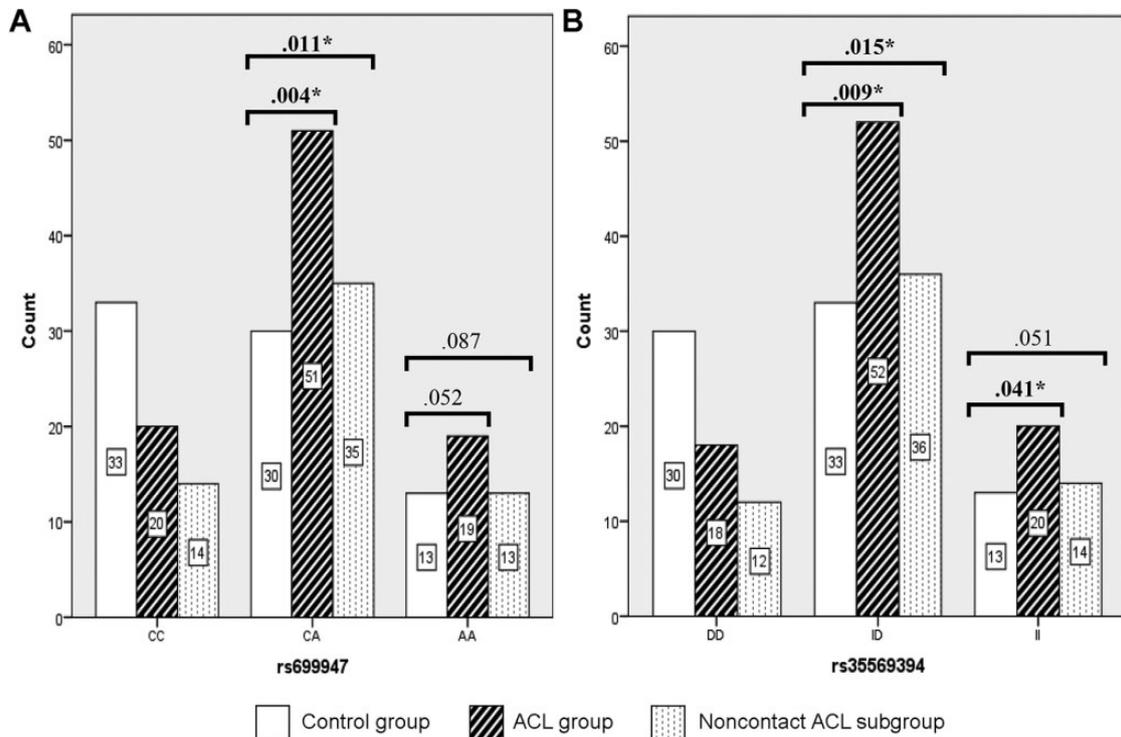
Figure 2. Genotype frequency distribution. (A) Vascular endothelial growth factor A (VEGFA) rs699947 among the control group, anterior cruciate ligament (ACL) group, and noncontact ACL subgroup. (B) VEGFA rs35569394 among the control group, ACL group, and noncontact ACL subgroup. *P values are significant at the .05 level.

TABLE 4
Haplotype Analysis and Linkage Disequilibrium for Common Haplotypes of VEGFA rs699947 and rs35569394

VEGFA rs699947	VEGFA rs35569394	Frequency	χ^2 Value	P Value	r^2 Value	D$'^a$	LOD Score^b
C	D	0.545	5.024	**.025**			
C	I	0.018	0.044	.834	**0.929**	**1.0**	63.74
A	I	0.437	5.320	**.021**			

^{a}Bolded values indicate statistical significance at 0.05 level. Theoretical range: 0 (complete linkage equilibrium) to +1 (complete linkage disequilibrium). VEGFA, vascular endothelial growth factor A.
^{b}LOD (logarithm of the odds) score >3 was considered significant linkage.

VEGFA (rs699947 C/A and rs35569394 18bp I/D) Haplotype Analysis and LD

Inferred haplotype analysis for VEGFA polymorphisms (rs699947 C/A and rs35569394 18bp I/D) revealed perfect LD between VEGFA rs699947 (-2578 C/A) and VEGFA rs35569394 (-2549 18bp I/D) (Table 4 and Appendix Figure A1). Patients who possess the C allele at position -2578 inside the VEGFA promoter (rs699947) have an 18-bp deletion at the -2549 position, resulting in enhanced VEGF protein expression.[4,7,12,19,27] We found that the C-D haplotype was significantly overrepresented in the control group, which suggests that enhanced VEGF protein expression helps to improve the healing capacity of musculoskeletal soft tissues after trauma and restores the biomechanical strength of the ACL.[29,30] The A-I haplotype was overrepresented in the ACL group.

Limitations

Although our study presents promising results for screening ACL injury–prone athletes and has scope in the individualization of a training program, there remain certain limitations of this study. In our experience, most athletes and their coaches did not agree with providing blood or oral mouthwash samples, or complying with other invasive sample collection methods, thus resulting in a limited number of participants for this study owing to administrative constraints and the fear of disclosure of their identity or doping sanctions. The limited number of female athletes

in the study may be attributed to the cultural issue of sports participation in India and to higher dropout rates among female athletes after ACL tears and other injuries, as female patients are considered to be at an increased risk of ACL ruptures; several studies have investigated sex-specific genetic associations with ACL injuries.[2,15,19,28,35] Also, patients were categorized into the contact or noncontact ACL group according to their self-reported mechanism of injury. As there is no current clinical tool to confirm the mechanism of ACL injuries, this remains a limitation of the study.

CONCLUSION

This was the first study to investigate the association of VEGF promoter polymorphisms with ACL injuries among Indian athletes. Multifactorial analyses of ACL injuries incorporating molecular profiles of angiogenesis-promoting, collagen matrix–remodeling, and repair-associated genes; gene-gene interactions; and linkage disequilibrium should be performed to fully elucidate the pathophysiology of sports injuries in general and ACL injuries in particular.

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APPENDIX

TABLE A1
Standard Genotyping Protocols Used for Screening Selected Polymorphisms[a]

Polymorphism	Screening Method	Primer Sequence	PCR Conditions
VEGFA rs699947	PCR-RFLP	Forward: 5′-ATAAGGGCCTTAGGACACCA-3′ Reverse: 5′-GCTACTTCTCCAGGCTCACA-3′	1. Initial denaturation: 94°C for 5 min 2. Denaturation: 95°C for 45 s 3. Annealing: 61°C for 30 s 4. Extension: 72°C for 45 s 5. Final extension: 72°C for 5 min Steps 2-4 repeated for 35 cycles
VEGFA rs35569394	PCR	Forward: 5′-CATTCCCATTCTCAGTCCAT-3′ Reverse: 5′-CCCATCCCATTCTTGCATA-3′	1. Initial denaturation: 94°C for 5 min 2. Denaturation: 95°C for 45 s 3. Annealing: 58°C for 30 s 4. Extension: 72°C for 45 s 5. Final extension: 72°C for 5 min Steps 2-4 repeated for 35 cycles

[a]PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; VEGFA, vascular endothelial growth factor A.

TABLE A2
2 × 3 Phenotype-by-Genotype Association Analyses of Selected VEGFA Polymorphisms With ACL Injury Risk Among Male Participants[a]

	Control (n = 59)	ACL (n = 75)	Control vs ACL		Noncontact ACL (n = 55)	Control vs Noncontact ACL	
			P Value	OR (95% CI)		*P* Value	OR (95% CI)
VEGFA rs699947							
CC	27 (46)	16 (21)	>.99	>0.99	12 (22)	>.99	>0.99
CA	23 (39)	41 (55)	**.006**	3.01 (1.34-6.71)	33 (60)	**.007**	3.23 (1.36-7.66)
AA	9 (15)	18 (24)	**.016**	3.38 (1.23-9.28)	10 (18)	.107	2.50 (0.81-7.73)
χ^2 value (*P* value)			9.09 (**.011**)			7.48 (**.024**)	
VEGFA rs35569394							
DD	24 (41)	14 (19)	>.99	>0.99	11 (20)	>.99	>0.99
ID	26 (44)	42 (56)	**.014**	2.77 (1.22-6.29)	31 (56)	**.032**	2.60 (1.08-6.30)
II	9 (15)	19 (25)	**.013**	3.62 (1.29-10.15)	13 (24)	**.039**	3.15 (1.04-9.56)
χ^2 value (*P* value)			8.17 (**.017**)			5.86 (.053)	

[a]Data are reported as n (%) unless otherwise indicated. *P* values are unadjusted. Bolded *P* values indicate statistical significance ($P < .05$). ACL, anterior cruciate ligament; OR, odds ratio; VEGFA, vascular endothelial growth factor A.

TABLE A3
Analysis of Selected Polymorphisms Based on 3 Genetic Models for ACL Injury Risk Among Male Participants[a]

| | Control | ACL | Control vs ACL | | Noncontact ACL | Control vs Noncontact ACL | |
			χ^2 Value (*P* Value)	OR (95% CI)		χ^2 Value (*P* Value)	OR (95% CI)
VEGFA rs699947							
Additive			7.37 (**.007**)	1.98 (1.21-3.25)		4.24 (**.039**)	1.75 (1.03-2.97)
C	77 (65)	73 (49)			57 (52)		
A	41 (35)	77 (51)			53 (48)		
Dominant (CC vs CA + AA)			9.04 (**.003**)	3.11 (1.46-6.61)		7.25 (**.007**)	3.02 (1.33-6.86)
C	27 (46)	16 (21)			12 (22)		
A	32 (54)	59 (79)			43 (78)		
Recessive (CC + CA vs AA)			1.57 (.210)	1.75 (0.72-4.25)		0.18 (.675)	1.23 (0.46-3.31)
C	50 (85)	57 (76)			45 (82)		
A	9 (15)	18 (24)			10 (18)		
VEGFA rs35569394							
Additive			6.84 (**.009**)	1.92 (1.17-3.14)		4.87 (**.027**)	1.80 (1.07-3.07)
D	74 (63)	70 (47)			53 (48)		
I	44 (37)	80 (53)			57 (52)		
Dominant (DD vs ID + II)			7.86 (**.005**)	2.99 (1.37-6.51)		5.72 (**.017**)	2.74 (1.18-6.36)
D	24 (41)	14 (19)			11 (20)		
I	35 (59)	61 (81)			44 (80)		
Recessive (DD + ID vs II)			2.03 (.154)	1.88 (0.78-4.54)		1.28 (.257)	1.71 (0.67-4.42)
D	50 (85)	56 (75)			42 (76)		
I	9 (15)	19 (25)			13 (24)		

[a]Data are reported as n (%) unless otherwise indicated. *P* values are unadjusted. Bolded *P* values indicate statistical significance ($P < .05$). ACL, anterior cruciate ligament; OR, odds ratio; VEGFA, vascular endothelial growth factor A.
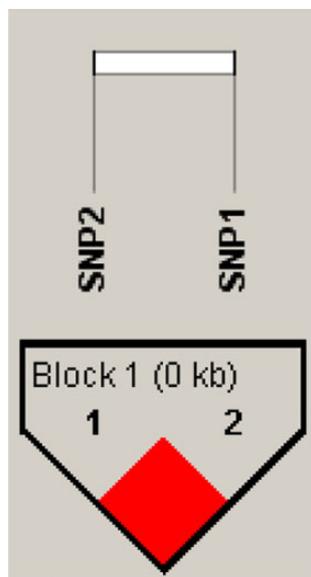

Figure A1. Pairwise linkage disequilibrium plot between vascular endothelial growth factor A polymorphisms rs699947 (single nucleotide polymorphism [SNP] 1) and rs35569394 (SNP 2). The red color shows a high r^2 value.