

Association of ACL tears and single nucleotide polymorphisms in the collagen 12 A1 gene in the Indian population - a preliminary case-control study

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Summary

Background: Genetic predisposition to ACL tears has received tremendous interest in the past few years with many SNPs of different genes being linked to ACL tear.

Study Objectives: To examine if specific sequence variants in COL12A1 gene are associated with ACL tears in Indian population.

Study design: Case-control study.

Materials and methods: 50 patients with surgically diagnosed ACL tear and 52 healthy, age-matched controls without any ligament/tendon injuries were genotyped for rs970547 and rs240736 SNPs using real time PCR method.

Results: The AG and GG genotypes were significantly under-represented in study group patients in rs970547 region ($p=0.0361$). However, there was no significant difference in genotype/allele frequencies in the rs240736 region.

Conclusions: The COL12A1 rs970547 SNP is associated with ACL tears in the Indian population. However, these results need to be validated further so that predisposed individuals can be screened in the future for counselling and intervention.

Level of evidence: III

KEY WORDS: ACL tear, COL12A1, gene polymorphisms, genetic association study, single nucleotide polymorphism (SNP).

Introduction

Anterior Cruciate Ligament (ACL) tears are now understood to have a multi-factorial etiology^{1,2}. Although trauma to the knee is an essential pre-requisite for an ACL tear, various risk factors (both extrinsic and intrinsic) have been identified, which predispose an individual to tearing his ACL^{1,2}.

Over the past few years, familial predisposition and specific single nucleotide polymorphisms (SNP's) of various collagen and extracellular matrix protein genes have been linked to ACL tear in different population subsets^{3,4}. However, most of these studies have focussed on Caucasian populations; till date, there are no published studies looking into the genetic risk factors of ACL tear from the Indian sub-continent^{3,4}.

Collagen XII is one of the main structural components of ACL collagen along with collagen types I, III-VI, XIV and various proteoglycans and glycoproteins⁵. The COL12A1 gene (mapped to chromosome 6q12-q13) encodes the $\alpha 1$ chains of the long (XIIA) and short (XIIB) homo-trimeric isoforms of type XII collagen⁶⁻¹⁰. Database hosted by the National Center for Biotechnology and Information (NCBI), has identified 5 SNPs in COL12A1 exonic regions of which only 2 (rs970547 and rs240736) have been identified as non-synonymous SNPs (*i.e.* SNPs that change the amino acid sequence in the gene product)¹¹. These 2 SNPs have been tested for association with ACL tears in South African and Polish populations with variable results^{12,13}.

The aim of our study was to determine if these 2 SNPs (rs970547 and rs240736) of the COL12A1 gene have any association with ACL tears in the Indian population.

Materials and methods

The study was conducted in the Department of Orthopaedics in collaboration with Neuroscience Research Laboratory at Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, India. Ethical clearance was obtained from the institute ethics committee prior to the commencement of the study. The study has been conducted ethically according to set international standards and meets the ethical standards of the journal¹⁴.

Selection of patients

Fifty patients with clinico-radiological and surgically proven ACL tears were recruited into the study group from July 2012 to December 2013. The inclusion criteria were: (i) age between 18-40 years, (ii) no multi-ligament injuries or signs of osteoarthritis knee (iii) no associated co-morbidities. Fifty-two age-matched patients with normal knees and no history of ligament/tendon injuries were recruited as controls. All subjects underwent thorough clinical evaluation. Informed, written consent was obtained from cases and controls. All subjects completed a detailed questionnaire consisting of general details, mode of injury, relevant past, personal and family histories along with details of participation in sports.

All procedures were conducted in a good laboratory practice (GLP) compliant laboratory utilising pre-calibrated instruments, quality assurance norms and as per established standard operating protocols (SOP's); documentation was done in DRS (display resource file) format by trained research scholars who were blinded to samples as prescribed by OECD (Organisation for economic co-operation and development) guidelines. External audit of results was carried out by seeking expert opinion from an outside laboratory.

DNA isolation

Five ml of venous blood was drawn into an EDTA vacutainer which was kept for around 2 hours at room temperature. Once the RBC's settled down the upper layer was aspirated and laid over equal amount of ficoll-paque solution (Amersham Biosciences, USA). After centrifugation at 1800 rpm for 30 minutes, a thick buffy coat of lymphocytes (peripheral blood monocytes/PBMC) found as a thin middle layer was collected in a fresh tube. The PBMC was pelleted by centrifugation at 5000 rpm for 5 minutes. The PBMC was then washed with PBS and processed for DNA isolation.

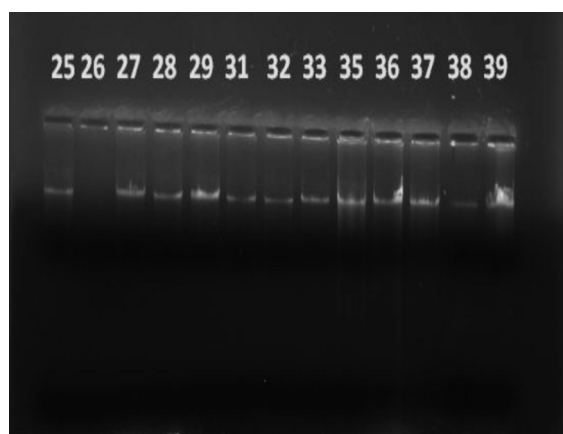


Figure 1. DNA isolated from PBMC electrophoresed on 1% agarose gel for quality verification.

Genomic DNA was isolated using QIAGEN DNeasy kit. The eluted DNA was quantified using UV spectrophotometer (Backman Coulter) and run on 1% agarose gel (Biorad) to verify the quality of DNA (Fig. 1).

COL12A1 genotyping

SNPs were analysed by using real-time PCR (polymerase chain reaction) performed in the 48 wells model Step one™ (Applied Biosystems Inc, Foster City, USA). Real time PCR was carried out for 20 μ L containing 10 μ L master mix, 5 μ L assay, 20 ng DNA and molecular biology grade water was added to make the volume 20 μ L. All reactions were carried out using TaqMan/SybrGreen SNP genotyping assays according to manufacturers' recommendations. Two reporter dyes-VIC and FAM were used to label the Allele 1 and 2 probes and a 5'Nuclease assay was carried out. Negative controls included the PCR mix without DNA. Software Step One™ v2.0 (Applied Biosystems, Foster City, USA) was used to perform amplification and to estimate SNPs. After PCR amplification, the Sequence Detection System (SDS) software was used to import the fluorescence measurements made during the plate read to plot fluorescence (Rn) values.

Statistical analysis

Data was analysed using SPSS software version 16.0 (Statistical Package for the Social Sciences). Normal-Quantile (Q-Q) plots were constructed in order to examine whether the data was normally distributed or not. For comparison of the 2 groups, unpaired student-t test was applied. Hardy Weinberg equilibrium was established prior to genotyping analysis. In RT-PCR, the genotypes for each mutation were stratified for heterozygosity, and homozygosity of the respective allelic variant. Pearson's Chi-square test followed by Cochran-Mantel-Haenszel equation was applied for the analysis of genotyping results.

Results

SNP rs970547

The AG and GG genotypes were significantly under-represented in study group patients ($p=0.0361$ and 0.0374 respectively; Tab. I, Figs. 2, 3). No significant difference between allele frequencies was observed ($p=0.091$). All groups were in Hardy Weinberg Equilibrium (HWE value=0.5).

SNP rs240736

There was no significant difference in the genotype ($p=0.712$) or allele frequencies ($p=0.4882$) between the 2 groups for the rs240736 region (Tab. II, Fig. 4). All groups were in Hardy Weinberg Equilibrium for this region (HWE value=0.02).

Table I. Effect of rs970547 variant on disease phenotype.

Genotype	Number	OR	95%CI	Z-statistics	p Value		
AA	ACL	Controls					
	10	3	Reference	-	-	-	
	AG	19	26	0.2192	0.053-0.9064	2.096	0.0361
	GG	15	21	0.2143	0.0502-0.9139	2.082	0.0374

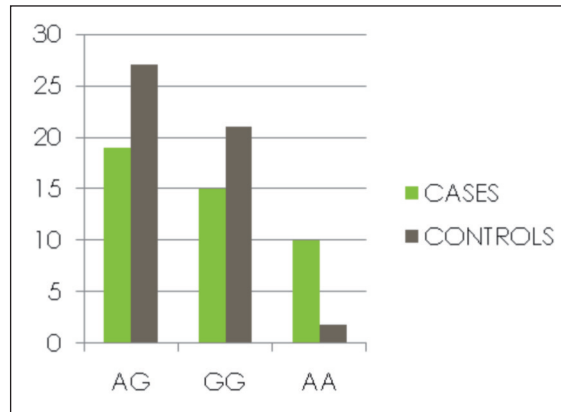


Figure 2. Bar diagram depicting relative frequency of genotypes of rs970547 in COL12A1 gene.

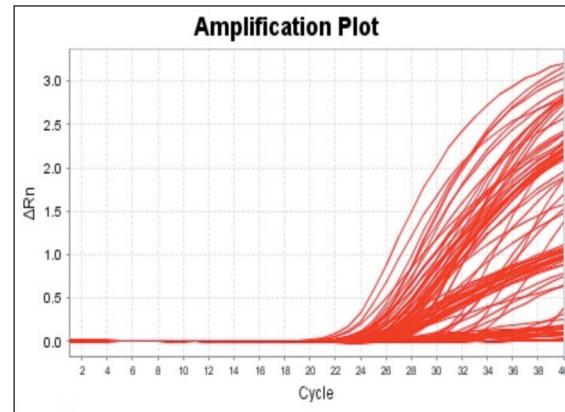


Figure 3. Amplification plot for ACL control and patient from SNP genotyping analysis for rs970547 variant.

Table II. Effect of rs240736 variant on disease phenotype.

Genotype	Number		OR	95%CI	Z-statistics	p Value
CC	ACL	Controls				
	28	28	Reference	-	-	-
CT	17	21	0.81	0.354-1.85	0.501	0.693
TT	0	1	*	*	*	*

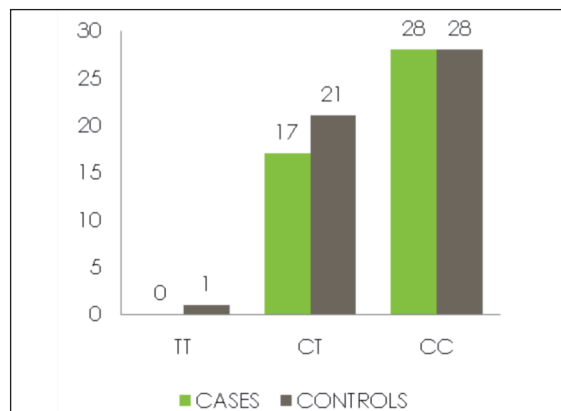


Figure 4. Bar diagram depicting relative frequency of genotypes of rs240736 in COL12A1 gene.

Discussion

Type XII collagen protein is associated with the surface of the collagen micro fibril and is a member of the Fibril Associated Collagens with Interrupted Triple helices (FACIT) sub-family. It has numerous functions: (i) involves in fibrillogenesis along with type XIV collagen, (ii) forms inter-fibrillar connections and mediates fibril interaction with other extracellular and cell surface molecules within ligaments there by influencing fibril and matrix density, (iii) regulates expression in response to mechanical loading, (iv) increases in content during healing along with type V and type III collagen, (v) regulates collagen fibril diameter (along with type V collagen) ⁵⁻¹⁰.

The association of COL12A1 SNP with ACL tear has been investigated previously in 2 Caucasian popula-

Table III. Summary of studies in literature investigating association of COL12A1 gene SNPs with ACL tears.

SI no.	Authors	Year of publication	Study type	No of ACL cases	No of controls	Population	Results
1	Posthumus et al. ¹¹	2010	Case control study	129	216	South Africa	Over-representation of AA genotype of rs970547 in female patients with ACL tears. No association of rs240736 with ACL tear.
2	Ficek et al. ¹²	2014	Case control study	91	143	Poland	No association of ACL tear with rs970547 SNP.
3	Our study	-	Case control study	50	52	India	Over-representation of AG & GG genotypes of rs970547 in ACL tear cases. No association of rs240736 with ACL tear.

tions^{12,13} Posthumus et al.¹² conducted a case-control genetic association study in South African participants (129 ACL tear patients and 216 healthy controls) to look for association between COL12A1 gene and ACL tears. They reported an over-representation of AA genotype of rs970547 (AluI RFLP) in female patients with ACL tears ($p=0.048$) but no significant association of the AA genotype with ACL tear when females and males were analysed together ($p=0.067$) or when males were analysed separately ($p>0.05$). However, rs240736 (BsrI RFLP) did not have any association with ACL tear in the South African cohort¹². The same research group had also noted the association of COL12A1 SNPs with Achilles tendon ruptures¹¹. The rare CC and GG genotypes of the COL12A1 rs240736 (BsrI RFLP) and rs970547 (AluI RFLP), respectively, were absent in participants with Achilles tendon ruptures¹¹.

Ficek et al.¹³ conducted a similar case-control genetic association study in the Polish population (91 male football players with ACL tears and 143 healthy, male football players all of Polish descent). They observed no statistically significant association of SNP rs970547 (A9285G polymorphism) with ACL tears¹³. Our study results demonstrate that the AG and GG genotypes of rs970547 of COL12A1 was significantly under-represented in the study group participants ($p=0.0361$ and 0.0374 respectively; Figs. 2, 3). This means that the presence of AG and GG genotypes of this SNP could be somehow protective towards an ACL tear in the Indian population. This is in contrast to the observations of Posthumus et al.¹² who noticed over-representation of AA genotype only in females and to the observations of Ficek et al.¹³ who noted no association of this SNP to ACL tear. On the other hand, our finding that SNP rs240736 is not associated with ACL tear is consistent with the findings of Posthumus et al.¹² who also reported no significant association between ACL tears and polymorphisms in rs240736 (BsrI RFLP).

The under-representation of these genotypes somehow results in an altered type XII collagen protein, which may lead to an alteration of the biomechanical properties of the collagen fibrils, thus resulting in structurally weaker collagen fibres; this in turn may predispose an individual to an increased risk of ACL tear¹². However, this hypothesis is speculative and needs further research before validation (Tab. III).

This is the first study from Asia looking into the role of COL12A1 gene polymorphisms in the etiology of ACL tear. We used real time PCR for genotyping similar to Ficek et al.¹³ whereas the RFLP method of genotyping was used by the South African research group¹². RT-PCR is technically easier to perform, allows rapid quantification and has a higher accuracy and reliability¹⁵.

We acknowledge some limitations of our study. The sample size is relatively small; hence these findings must be interpreted with caution. Further research with a larger sample size is needed which will not only validate these findings but will also facilitate haplotype analysis. We could not comment on the differences in the genotype/allele distributions between male and female groups owing to lesser number of female participants. Gender-specific association, however, was not a primary objective of this study. Future studies should ideally include more number of female participants in both groups to discern any gender-specific associations between SNPs and ACL tear in the Indian population.

Conclusions

AG and GG genotypes in exon 65 of COL12A1 are associated with ACL tears in the Indian population. Thus, type XII collagen may be a good candidate for genetic screening of predisposed individuals, so that these individuals may be counselled, strategies may be employed to prevent ACL tear and in the future may be a potential target for gene therapy.

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