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Interactions Between *COL5A1* Gene and Risk of the Anterior Cruciate Ligament Rupture

by

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Collagen alpha-1(V) chain, encoded by the COL5A1 gene, plays a crucial role in abundant fibrillar collagens supporting many tissues in the body containing type I collagen and appears to regulate the association between heterotypic fibers composed of both type I and type V collagen occurring among others in muscles, tendons and ligaments. Taking this fact into consideration we decided to examine the association between COL5A1 rs12722 and rs13946 polymorphisms, individually and as inferred haplotypes, with anterior cruciate ligament rupture risk (ACLR) in professional soccer players. A total of 134 male professional soccer players with surgically diagnosed primary anterior cruciate ligament ruptures and 211 apparently healthy male professional soccer players, who were without any self-reported history of ligament or tendon injury, were included in the study. Both the cases and the healthy controls were recruited from the same soccer teams, of a similar age category, and had a comparable level of exposure to anterior cruciate ligament injury. Genomic DNA was extracted from oral epithelial cells using GenElute Mammalian Genomic DNA MiniprepKit. All samples were genotyped for the rs12722 and rs13946 polymorphisms using a Rotor-Gene real-time polymerase chain reaction. Statistically significant differences in the genotype frequencies for the COL5A1 rs13946 polymorphisms in dominant modes of inheritance occurred (p = 0.039). Statistically significant differences were documented only in the dominant model under the representation tendency of the C-C haplotype in the ACLR group compared to controls (p = 0.038). Our results suggest that variation in the COL5A1 gene may be one of the non-modifiable factors associated with the ACL injury in professional soccer players. The C-C rs12722-rs13946 haplotype provides a protective effect against the ACL tear.

***Key words**:* COL5A1, ACLR, soccer players, injuries.

Introduction

The *COL5A1* gene, localized on chromosome 9q34.3, encodes the alpha-1(V) chain of type V collagen. This minor fibrillar collagen plays acrucial role in the regulation of the size and configuration of other abundant fibrillar collagens supporting many tissues in the body, such as tendons, ligaments, and muscles (Birk at al., 1990).

It has been shown that mutation in the

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COL5A1 gene results in a 50% reduction of type V collagen and leads to poorly organized fibrils, decreased tensile strength, and reduced stiffness of connective tissue (Wenstrup et al., 2006). In addition, mutations within *COL5A1* were implicated in Ehlers Danlos syndrome (Malfait et al., 2010; Myllyharju and Kivirikko, 2001), a condition characterized by joint hypermobility. The *Bst*UI RFLP (rs12722) is a common C to T single nucleotide polymorphism (SNP) within the *COL5A1* 3′ untranslated region which may alter *COL5A1* messenger RNA (mRNA) stability (Laguette et al., 2011). Previous studies identified the *Bst*UI restriction fragment length polymorphism (RFLP) to be associated with chronic Achilles tendinopathy (Mokone et al., 2006; September et al., 2009), anterior cruciate ligament ruptures (O`Connell et al., 2015; Posthumus et al., 2009; Petr et al., 2014), carpal tunnel syndrome (Burger et al., 2015) and properties of the knee ligament (Kubo et al., 2013). In addition, this SNP was also associated with joint flexibility (Brown et al., 2011), joint range of motion (ROM) (Collins et al., 2009) and endurance running performance (Brown et al., 2011; Collins and Posthumus, 2011). It was hypothesized that individuals with the rs12722 TT genotype have increased type V collagen production and thus favorably altered mechanical properties of tendons, which potentially enhances endurance running ability (Collins et al., 2011; Posthumus et al., 2011). Moreover, individuals with the *COL5A1* T functional allele are reported to have an increased mRNA stability *in vitro* (Laguette et al., 2011). In these investigations, the TT genotype has been prominent with phenotypes of stiffer tendon and ligamentous mechanical properties (Laguette et al., 2011).

Mokone et al. (2006) reported a significant difference in the allele frequencies of the *COL5A1* rs13946 DpnII RFLP between the Achilles tendon pathology group and control groups). However, research investigating a group of recreational skiers showed no significant differences in rs13946 (C/T) genotype frequency distributions between the ACL rupture and control groups (Posthumus et al., 2009; Stępień-Słodkowska et al., 2015).

The aim of this study was to investigate the rs12722 and rs13946 polymorphisms in the *COL5A1* gene, individually and as haplotypes, with the risk of ACL ruptures in soccer players. We postulated that the *COL5A1* rs12722 and rs13946 polymorphisms would be individually associated with ACL rupture risk and that an inferred haplotype of the two polymorphisms may provide more information into the potential role of *COL5A1* in predisposing professional soccer players to higher risk of ACL rupture (O'Connell et al., 2015).

Methods

This study was approved by the Gdansk Medical University Ethics Committee, Poland (number KB 8/16) and written informed consent was obtained from each participant according to the declaration of Helsinki. A total of 134 professional male soccer players (age = 23.4 ± 3.1 years), with surgically diagnosed primary ACL ruptures who qualified for ligament reconstruction, were recruited for this study. All players had non-contact ACL ruptures. For the obvious reason that the soccer teams were homogenous in term of gender, we recruited only male subjects. All participants were soccer players playing in the Polish 1st division professional soccer league, with overall training time of 14–18 h per week (7–9 training sessions a week, 2 h each). Subjects were treated in the Galen Orthopaedics Clinic in Poland.

The control group consisted of 211 apparently healthy, male professional soccer players (age = 25.3 ± 3.4 years), without any self-reported history of ligament or tendon injury. Both the ACL rupture group and the healthy controls were from the same soccer teams, of the same ethnicity (all self-reported Polish, East-Europeans for ≥3 generations), and had a comparable level of exposure to risk of ACL injury (same volume and intensity of training as well as hours of match play).

We followed the STREGA recommendations for genotype-phenotype association studies (Little et al., 2009). An oral epithelium was collected from each participant using the DNA Swab (Copan, USA). Genomic DNA was extracted from the oral epithelial cells using a Gen Elute Mammalian Genomic DNA Mini prep Kit (Sigma, Germany) according to manufacturer's recommendations. Allelic discrimination of the *COL5A1* rs12722 and rs13946 polymorphic sites (*Bst*UI RFLP C/T and *Dpn*II

RFLP C/T, respectively) was performed using catalogued TaqMan® SNP Genotyping Assays (Applied Biosystems, USA). All samples were genotyped on a Rotor-Genereal-time polymerase chain reaction (PCR) instrument (Corbett, Australia) with the following cycling conditions: an initial hold step at 95°C for 5 min, followed by 45 cycles of denaturation at 94°C for 15 s and anneal/extend at 60°C for 1 min. 47 positive (known genotypes) and negative controls (no DNA) were included on each PCR plate as quality control measures. Genotyping results were called by two independent, experienced investigators who were blinded to the participants' data.

Statistical Analysis

The programming language and environment R (http://www.r-project.org) was used for all statistical analyses. Genotype and allele frequencies were compared between the cases and controls using χ^2 or Fisher exact tests. Allelic-based odds ratios (OR) with 95% confidence intervals (95%CI) were calculated using logistic regression analysis. The genotypes between cases and controls were compared in three ways: first, in a general test of association in the 2-by-3 table of phenotype-by-genotype, then two different modes of inheritance of the minor allele were assumed: dominant, in which homozygotes and heterozygotes for the minor allele were pooled and compared to homozygotes for the major allele and recessive, in which homozygotes and heterozygotes for the major alleles were pooled and compared to homozygotes for the minor allele. Hardy–Weinberg equilibrium probabilities and linkage disequilibrium (LD) between the SNPs were also calculated. The *haplo.stats* package was used to infer haplotype frequencies and to test the association between inferred haplotypes and the risk of ACL rupture assuming three possible haplotype effects: additive, dominant and recessive. *Hap.score* is the statistical co ref or haplotypes reflecting the strength of association; the positive value of *Hap.score* indicates increased risk of ACL injury for a particular haplotype, while a negative value indicates reduced risk. For all tests, significance was set at $p < 0.05$.

Table 1

COL5A1 rs12722 C/T and rs13946 C/T allelic and genotypic frequency distributions in the anterior cruciate ligament rupture group (ACL rupture group) in comparison with the control group.

SNP		**CON (n=211)**	**ACL (n=134)**	*p*
***COL5A1* rs12722**	TT	62 (29%)	45 (34%)	0.661
	CT	107 (51%)	66 (49%)	$p_D = 0.411$
	CC	42 (20%)	23 (17%)	$p_R = 0.526$
	C allele	191 (45%)	112 (42%)	0.371
	HWE	0.729	0.888	
***COL5A1* rs13946**	TT	94 (45%)	75 (56%)	0.091
	CT	102 (48%)	49 (37%)	$p_D = 0.039$
	CC	15 (7%)	10 (7%)	$p_R = 0.902$
	C allele	132 (31%)	69 (26%)	0.119
	HWE	0.071	0.610	

p_D and p_R are two-sided Fisher's exact test probabilities for dominant (CC+CT vs TT) and recessive (CC+CT vs TT) modes of inheritance of the minor allele (rs12722 and rs13946), respectively. HWE: hardy Weinberg exact tests of significance

Table 2

Analysis of the frequency distributions of the COL5A1 rs12722-rs13946 haplotype in the study group and the control group for the three genetic models - dominant, recessive and additive.

(Haplotype (rs12722-rs13946	Control (n=211)	Study (n=134)	Dominant model (global-stat = 4.35, p = 0.226)	Recessive model (global-stat = 4.72, p = 0.193)	Additive model (global-stat = 2.75, p = 0.253)
T-T	55%	58%	0.63, p = 0.525	0.82, p = 0.411	0.90, p = 0.366
C-C	31%	26%	-2.06, p = 0.038	0.12, p = 0.901	-1.60, p = 0.107
C-T	14%	1%	0.32, p = 744	1.90, p = 0.057	0.76, p = 0.442

global-stat –association of all haplotypes of the trait; hap.score – measure individual haplotype association to the trait; Significant p-values are in bold (p<0.05).

Results

At baseline, male professional soccer players with surgically diagnosed primary ACL ruptures and the control group did not differ significantly according to age and the level of exposure to ACL injury. The genotype and allele frequencies for the *COL5A1* rs12722 and *COL5A1* rs13946 are shown in Table 1. The genotype distributions for both polymorphisms met Hardy-Weinberg expectations in both groups.

There were no significant differences in the genotype or allele frequency distribution for either *COL5A1* (rs12722) or *COL5A1* (rs13946) polymorphisms between the control group and the ACL rupture group using the 2-by-3 general test of association (Table 1). Likewise, there were no significant differences in the genotype frequencies for the *COL5A1* rs12722 polymorphisms, when dominant and recessive modes of inheritance were assumed. However, significant differences in the genotype frequency distribution were noted for the *COL5A1* rs13946 polymorphisms when a dominant mode of inheritance was tested (p = 0.039) (Table 1).

Evaluation of the haplotype frequency distribution for *COL5A1* rs12722-rs13946 showed significant differences between the control group and the study participants. There were three haplotypes, T-T, C-C and C-T with frequencies of 56%, 29% and 15%, respectively. Specifically the C-C haplotype was found to be overrepresented in the control group compared to the ACLR group (p = 0.038) (Table 2) when the dominant model was tested.

Discussion

Recent genetic research has summarised the specific markers to be associated with increased risk for sports injuries (Collins et al., 2015; Rahim et al., 2016; September et al., 2016) and performance-related conditions (Cupeiro et al., 2010; Maffulli et al., 2013; Wang et al., 2013). Use of this genetic information can aid in the identification of the biological pathways underpinning injury risk and may, in future, support the development of tailored injury prevention programmes for athletes and the application of targeted therapeutic interventions (Cięszczyk et al., 2017).

In our study, we examined the association between both *COL5A1* rs12722 C/T (*BstUI* RFLP) and the *COL5A1* rs13946 C/T (*DpnII*) polymorphisms individually and as haplotypes with anterior cruciate ligament rupture risk in professional soccer players. Our findings identified no significant differences in the genotype (under general model of association) and allele frequency distributions of any of these two polymorphisms between the control group and the ACL rupture group. However, the carriers of the rs13946 C allele were underrepresented in the ACL injury group compared with controls. Considering the lack of association in the allele-based test, our results are consistent with those previously reported by Stępień-Słodkowska et al. (2015) in recreational skiers from Poland. Similarly, we identified the T-T haplotype (*COL5A1* rs12722 C/T-rs13946 C/T) to be the most common (56%). The similarities in the frequency distributions between the two independent studies may be a result of both study groups originating from Poland. Further research in larger sample sizes and in independent populations is needed to investigate the role of the rs12722 and the rs13946 polymorphisms in ACL rupture risk.

Previously the rs12722 CC genotype was found to be associated with ACL tears in females in a Caucasian study group from South Africa (Posthumus et al., 2009). In addition, the rs12722 SNP was also associated with the development of bilateral quadriceps tendon rupture (Longo et al., 2010) and both the rs12722 and rs13946 SNPs were identified as genetic risk factors for tennis elbow (Altinisik et al., 2015)

In this study we found significant differences in the genotype frequencies for the *COL5A1* rs13946 polymorphisms in dominant modes of inheritance, underrepresentation tendency of the C-T haplotype in the ACLR group compared to controls.

However, the authors do note that the sample size in both studies is a major limitation for such genetic association study. It is therefore recommended that the number of subjects be increased for a more comprehensive risk profiling analysis for ACLR susceptibility.

Several additional polymorphisms within *COL5A1* have also been associated with exercise-related phenotypes and are good candidates for further investigation. The *MIR608* rs4919510 CC genotype was significantly over-represented in tendinopathy participants compared to asymptomatic controls (Laguette et al., 2011). Interestingly, the rs4919510 SNP (on chromosome 10q24) was associated with the functional microRNA (miRNA) binding site for Hsa-miR-608 within the *COL5A1* 3′-UTR, thereby affecting *COL5A1* mRNA stability *in vitro*. Hsa-miRNA-608 binds to a functional polymorphic cis-acting element within the *COL5A1* 3′-UTR (Abrahams et al., 2013; Laguette et al., 2011). Kirk et al. (2016) indicated that the *COL5A1* rs1536482 (A/G) and rs12722 (C/T) may potentially influence quadriceps muscle-tendon stiffness, but not low-level contractile properties.

Conclusions

Our results suggest that variation in the *COL5A1* gene may be one of the non-modifiable factors associated with the ACL injury in professional soccer players. The C-C rs12722-rs13946 haplotype provides a protective effect against the ACL tear.

Practical Implications

Although there are no immediate clinical implications, these findings help identify the main biological pathways contributing to injury susceptibility. In future, these genetic risk factors could be included in multifactorial risk models to assess one's ACL rupture susceptibility. Moreover, this information could support the development of tailored injury prevention programmes and/or targeted therapeutic interventions.

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