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Association of the *COL27A1* rs946053 and *TNC* rs2104772s with Tendinopathies: Case-Control Study in High-Level Athletes

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Abstract

Background/Objectives: Increased risk of developing tendinopathies in athlete population has led to investigations of several genes associated with tendon properties, suggesting some individuals have greater genetic predisposition for developing tendinopathies. The main purpose of this study was to investigate how the functional polymorphisms within the *COL5A1*, *COL27A1* and *TNC* genes impact risk of developing tendinopathies in Croatian high-level athletes. **Methods**: For this case-control genetic study we have recruited sixty-three high-level athletes with diagnosed tendinopathies and ninety-two asymptomatic healthy controls, all being unrelated Caucasians. Participants were genotyped for three single nucleotide polymorphisms (SNP) within *COL5A1*, *COL27A1* and *TNC* genes using the pyrosequencing method. **Results**: *TNC* rs2104772 TT (P=0.0089) and T-T-T haplotype (P=0.0234) constructed of rs12722, rs946053 and rs2104772 were significantly over-represented in cases versus controls, implicating predisposition for tendinopathies. *COL27A1* rs946053 GG (P=0. 0118) and G-A-C haplotype (P=0.0424) constructed of rs12722, rs946053 and rs2104772 were significantly over-represented in controls implicating protective role. **Conclusions**: These results further support associations between functional polymorphisms within the *COL27A1* and *TNC* genes and risk of tendinopathies in high-level athletes. Further research is needed to replicate these results in various populations and in larger cohorts.

Keywords: collagen 27 alpha1, tenascin C, soft tissue injury (STI), single nucleotide polymorphism (SNP), genetic association study, achilles tendinopathy, sport injury

1. Introduction

Regular physical activity benefits those who participate, even though it also increases injury risk. Sedentary lifestyle usually led by recreational athletes prior to returning to a regular sport activity is often underlying cause of sudden overuse injuries that affect tendons, while tendon ruptures and tendinopathies are serious injuries affecting athletes, causing pain and disfunction and adding to the delay in recovery and prolonging return to competing for professional athletes [1,2]. Tendon role is to facilitate smooth joint movements, but through that process tendons sustain large strain when transmitting force from muscle to bone. Achilles tendon ruptures are higher in males than in females, with ratio ranging from 2:1 to 12:1 [3,4], just like the prevalence of Achilles tendinopathy seems to be increasing through the population, ranging from 10% in general population to up to 50% within professional athletes [5,6]. Tendinopathies are common overuse injuries associated with sports, in acute or chronic states, mostly affecting Achilles, patellar, rotator cuff and forearm extensor tendons [7]. Different extrinsic and intrinsic factors have been reported. Extrinsic factors include occupation,

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sport, physical load, training errors and some other factors, while intrinsic factors include age, gender, nutrition, anatomical variants, joint laxity and genetic susceptibility [8,9]. Tendons are highly ordered in structure, made of tightly packed bundles of fibrils. Collagen is the most abundant protein in tendons making up to 80% of total dry mass. Glycoproteins, proteoglycans, such as elastin and tenascin C and other proteins make rest of the tendon dry mass. Type I collagen makes most of the collagen dry mass, while type III and type V collagen and few others add to the rest [10]. Type V collagen, encoded by the *COL5A1* gene, regulates diameter of type I collagen fibrils, consists of the triple α1 chains and appears in tissues where collagen type I is expressed [11,12]. The *COL5A1* gene is located on the long arm of chromosome 9 (9q32-q34). Single nucleotide polymorphisms (SNPs) occur normally throughout the genome, on average in every 100-300 base pairs and add up to 90% of the changes within the genome, resulting in roughly 4 to 5 million SNPs. Several SNPs have been found and associated with Achilles tendon injuries, anterior cruciate ligament rupture and range of motion measurements [13-17]. Just like the *COL5A1*, the *TNC* gene is too located on the chromosome 9, 19.6 Mbp upstream of the *COL5A1*, encoding the tenascin C glycoprotein, expressed in highly constrained manner in embryonic tissues, as well as in adult tissues during remodeling and would healing [18,19]. The extracellular matrix of musculoskeletal tissues that transmit mechanical forces and are exposed to high stress have high levels of TNC expression [20]. TNC glycoprotein plays an important role in regulation cell-matrix interactions. Polymorphisms within the *TNC* gene have been associated with Achilles tendinopathy [21,22], allergic diseases [23] and adult asthma [24]. Another gene in the proximity of *TNC* and *COL5A1* may be of interest. The *COL27A1* gene encodes the homotrimeric type XXVII fibrillar collagen, that provides structural framework and tensile strength and is highly conserved in vertebrates [25,26]. The risk of Achilles tendinopathy was significantly associated for the haplotype consisting of the *TNC* and *COL27A1* sequence variants in Caucasian population [27]. The aim of this study was to investigate a possible association between the *COL5A1* rs12722, *COL27A1* rs946053 and *TNC* rs2104772 polymorphisms and tendinopathies in Croatian athletes. The hypothesis of this study is that polymorphisms are associated with the incidence of tendinopathy occurrence and that there will be a difference in genotype frequency and haplotypes between affected and athletes that were not affected.

2. Materials and Methods

Participants

One hundred and fifty-five participants were recruited between 2016 and 2017 and included in a case-control genetic association study. All participants are self-reported unrelated Caucasians and physically active athletes of various sports. The Achilles tendinopathy group (TEN) had clinically diagnosed Achilles tendinopathy, with symptoms including progressive pain, tendon swelling or changes of the lesions thickness and sensitivity to palpation. TEN group consisted of 63 participants (47 male and 16 female, average age 32 years), most being high level competing athletes in their prospective sports, with 3-7 coach supervised trainings per week. TEN participants were recruited in Orthopedic Clinic by their team medical doctor, orthopedic surgeon. Control group (CON) consisted of 92 participants (72 male and 20 female, average age 39.0 years) that were done with their active athlete career and have self-reported not having tendinopathies during their active competing career. This study was approved by the Ethics Committee of the School of Medicine, University of Zagreb, Croatia and by the Ethics Committee of the Faculty of Kinesiology, University of Zagreb, Croatia. All participants have provided a biological sample and given written informed consent.

Genotyping

Genomic deoxyribonucleic acid (DNA) was extracted from the oral epithelial cells using QIAamp DNA kit (Qiagen, Germantown, MD, USA). Polymerase chain reaction (PCR) amplification was conducted using COL5A1 rs12722, COL27A1 rs946053 and TNC rs2104772 T>A specific primer pairs (Metabion, Planegg/ Steinkirchen, Germany) and PyroMark PCR Kit (Qiagen, Germantown,

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MD, USA). Amplified fragments were sequenced by pyrosequencing method (PyroMark Q24, Qiagen, Germantown, MD, USA) according to the manufacturer's protocol and analyzed by PyroMark Q24 software (Qiagen, Germantown, MD, USA).

Statistical analysis

Allelic, genotypic and haplotype differences were anaalysed using an odds ratio method, Statcalc program (AcaStat software, Orange County, FL, USA). When P was $P<0.05$ it was considered being a statistically significant difference. For haplotype analysis we used Phase software (Matthew Stephens Laboratory, University of Chicago, IL, USA). The Hardy–Weinberg equilibrium analysis was performed using Genetics package for R software. In this study we used groups similar in size to previously reported studies [13,15,28] investigating genotype effects on various soft-tissue injuries, as those group sizes proved to be large enough to detect significant results. The Bonferroni correction is considered too conservative [29], so it was not applied in this study. P values were adjusted for false discovery rate (FDR) using Benjamini-Hochberg procedure for adjusted P value. It was applied for each genotypic, allelic and haplotype separately.

3. Results

Participants entering this study were divided in two groups: TEN group with diagnosed tendinopathy and CON group with no prior self-reported tendinopathy injuries during their active competing period in their professional career. It is important to notice that CON group has significantly higher weight and body mass index (BMI) and is chronologically older than TEN group, which is explained by the changes in lifestyle once professional athletes are retired. Average height of both TEN and CON group is comparable, and for the purpose of this study it was assumed that weight and BMI of the CON group at the peak point of retired athletes' career were comparable to TEN group. Data presented in Table 1. are matched for height, gender and ethnicity, not for BMI and weight as explained earlier.

Table 1. General characteristics of the tendinopathy group and control group.

	TEN (n=63)	Controls (n=92)	P-value
Age (years)	32.1 ± 12.8	39.0 ± 11.4	**0.006**
Height (cm)	180.5 ± 8.8	179.8 ± 9.6	0.6454
Weight (kg)	79.4 ± 14.9	84.6 ± 15.4	**0.0381**
BMI (kg/m2)	24.1 ± 3.6	26.0 ± 3.3	**0.009**
Ethnicity (Caucasian)	100% (63)	100% (92)	1.0000
gender (% male)	75% (47)	78% (92)	0.6639

Ethnicity and gender are represented as a percentage, the remaining variables are expressed as a mean ± standard deviation. Significant P-values are in bold. Age, height and weight are self-reported values in time of the recruitment.

Genotype frequencies were significantly different between TEN and CON groups. GG genotype of rs946053 was over-represented in controls when compared with cases, therefore classified as protective, while TT genotype of rs2104772 T>A was significantly over-represented in TEN group and is associated with the higher risk of tendinopathies. None of the rs12722 genotypes has any significant relevance in our cohort. In the similar manner, allelic frequencies were significantly different between two groups. G allele of rs946053 and rs2104772 A allele where significantly over-represented in controls and determined as protection, while T alleles of both rs946053 and rs2104772 were significantly over-represented in cases when compared to controls, so they are considered a predisposition for tendinopathies development. T and C allele of rs12722 did not show associations.

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All polymorphisms conformed to the Hardy-Weinberg equilibrium (HWE) in both cases and control group (Table 2).

Table 2. Allele and genotype frequency.

COL5A1				Allele frequency				
SNP rs12722	C>T	TEN n=63	CON n=92	p	FDR	OR	95% CI	Association
1	C	42.1% (53)	45.1% (83)	0.5957		0.8835	0.5590 - 1.3962	none
2	T	57.9% (73)	54.9% (101)	0.5957		1.1319	0.7162 - 1.7888	none
				Genotype frequency				
SNP rs12722	C>T	TEN n=63	CON n=92	p	FDR	OR	95% CI	Association
11	CC	14.3% (9)	21.7% (20)	0.2456		0.6000	0.2533 - 1.4210	none
12	CT	55.6% (35)	46.7% (43)	0.2816		14.244	0.7481 - 2.7121	none
22	TT	30.1% (19)	31.6% (29)	0.8570		0.9381	0.4682 - 1.8795	none
HWE			0.267					
COL27A1				Allele frequency				
SNP rs946053	G>T	TEN n=63	CON n=92	p	FDR	OR	95% CI	Association
1	G	43.7% (55)	56.5% (104)	**0.0264**	0.0264	0.5959	0.3773 - 0.9412	**protection**
2	T	56.3% (71)	43.5% (80)	**0.0264**	0.0264	1.6782	1.0625 - 2.6506	**Predisposition**
				Genotype frequency				
SNP rs946053	G>T	TEN n=63	CON n=92	p	FDR	OR	95% CI	Association
11	GG	14.3% (9)	32.6% (30)	**0.0118**	0.0354	0.3444	0.1503 - 0.7895	**protection**
12	GT	58.7% (37)	47.8% (44)	0.1829		15.524	0.8127 - 2.9656	none
22	TT	27% (17)	19.6% (18)	0.279		15.193	0.7118 - 3.2428	none
HWE			0.124					
TNC				Allele frequency				
SNP rs2104772	T>A	TEN n=63	CON n=92	p	FDR	OR	95% CI	Association
1	T	61.1% (77)	48.4% (89)	**0.0276**	0.0276	1.6774	1.0586 - 2.6579	**predisposition**
2	A	38.9% (49)	51.6% (95)	**0.0276**	0.0276	0.5962	0.3762 - 0.9447	**protection**
				Genotype frequency				
SNP rs2104772	T>A	TEN n=63	CON n=92	p	FDR	OR	95% CI	Association
11	TT	42.9% (27)	22.8% (21)	**0.0089**	0.0267	25.357	1.2628 - 5.0918	**predisposition**
12	TA	36.5% (23)	51.1% (47)	0.0745		0.5505	0.2857 - 1.0608	none
22	AA	20.6% (13)	26.1% (24)	0.4351		0.7367	0.3402 - 1.5869	none
HWE			0.066					

Allele and genotype frequencies are expressed as percentage with the number of participants (n) in parentheses. Results that have been classified as significant by the P-value (P<0.05) are in bold and have been additionally checked for false discovery rate (FDR), using Benjamini-Hochberg adjusted P value. OR- odds ratio. 95% CI – 95% confidence interval.

Haplotypes of all possible combinations of SNPs rs12722, rs946053 and rs2104772 T>A were constructed and analysed, and after correcting for FDR, T-T-T haplotype was considered as predisposition for development of tendinopathies, while G-A-C haplotype was considered as protective, presented in Table 3.

Table 3. Haplotype frequency.

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Hap code	Haplotype	CON	TEN	p	FDR	OR	95% CI	Association
1	G - A - T	16	5	0.1038		0.4339	0.1547 - 1.2166	none
2	**G - A - C**	25	8	**0.0424**	**0.0424**	0.4310	0.1880 - 0.9900	**protection**
3	G - T - T	32	21	0.8678		0.9500	0.5193 - 1.7380	none
4	G - T - C	31	21	0.9666		0.9871	0.5379 - 1.8114	none
5	T - A - T	42	30	0.7729		1.0565	0.6186 - 1.8046	none
6	T - A - C	11	6	0.6441		0.7864	0.2831 - 2.1843	none
7	**T - T - T**	11	17	**0.0234**	**0.0424**	2.453	1.107 - 5.434	**predisposition**
8	T - T - C	16	18	0.1219		1.7500	0.8556 - 3.5792	none

Results that have been classified as significant by the P-value (P<0.05) are in bold and have been additionally checked for false discovery rate (FDR), using Benjamini-Hochberg adjusted P value. OR- odds ratio. 95% CI – 95% confidence interval.

4. Discussion

Our understanding of the molecular mechanisms underlying soft tissue injuries is still limited. Different candidate gene variants are researched daily. Some of the previously reported variants have been successfully replicated in another populations, while some have stayed significant for one population only. Considering previous studies conducted on other populations, this study has investigated further variations within *COL5A1*, *COL27A1* and *TNC* genes and related risks of developing tendinopathies. The main finding of our study conducted in a cohort of Croatian competing athletes suggested that TT genotype of *TNC* rs2104772 T>A and T-T-T haplotype constructed of *COL5A1* rs12722, *COL27A1* rs946053 and *TNC* rs2104772 T>A had a significant association with the risk of developing tendinopathies. On the other hand, GG genotype of *COL27A1* rs946053 suggested protection of developing tendinopathies, as well as G-A-C haplotype constructed of *COL5A1* rs12722, *COL27A1* rs946053 and *TNC* rs2104772 T>A.

Many previous studies have suggested that genetic factors with the strongest evidence of association involved polymorphisms within *COL5A1*, *COL27A*, *TNC* genes, as well as matrix metaloproteinase-3 (MMP3) and estrogen-related receptor beta (ESRRB) [14-17,21,27,30,31].

September *et al.* investigated *COL5A1* gene and showed that individuals with CC genotype of rs12722 were predisposed to Achilles tendon injuries in South African and Australian population [14]. Following these findings, Brown *et al.* investigated *COL5A1* rs12722 further in the British cohort, but similarly to our own, in European cohort CC genotype was not significant in AT pathology. Although *COL5A1* rs12722 was not significantly overrepresented in AT group by itself, three inferred allele combinations constructed of rs12722, rs3196378 and rs71746744 within the *COL5A1* gene were identified as risk modifiers [32]. Study conducted on the population of young academic soccer players connected CC genotype and C-allele carriers for *COL5A1* rs12722 with predisposition to more soft tissue and ligament injuries, indicating that these associations depend on maturity status due to phase of physical development of these tissues [33]. Other than age, some other factors should be taken in account when identifying risk connected to genetic variants such as gender and ethnicity. Figueiredo *et al.* in their study on rotator cuff tear showed that C/T haplotype for *COL5A1* rs3196378 and rs11103544 has protective effect but only for males [34].

Contrary to these studies, Heffernan *et al.* observed large cohort of elite rugby players and associate C allele of rs12722 and rs3196378 with protective properties against tendon injuries, lower incidence of muscle cramping, and also reported generally greater frequency of allele C in players compared to control group [35].

Saunders *et al.* genotyped Australian and South African population for four polymorphisms within the *COL27A1* gene (rs946053, rs753085, rs1249744, rs4143245) and three within *TNC* gene (rs2104772, rs1330363, rs13321) resulting in the finding that the GCA haplotype (rs946053-rs13321-rs2104772) occurred significantly more frequent in TEN population [27]. Continuing in that direction, they investigated further implications of variants in several genes including *COL27A1* and *TNC*, as

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well as *IL-6*, *IL-1β* and *CASP8*, concluding there are subtle effects on protein signaling, interactions or alternate splicing that may be contributing to Achilles tendon pathologies [22].

Further research focused on a whole-exome sequencing approach, where Gibbon *et al.* sequenced ten healthy controls and ten patients with Achilles tendinopathy, by using a platform which included coverage of the untranslated regions as well as miRBase miRNA genes. Results showed four variants in *TNC* (rs1061494, rs1138545, rs2104772 and rs1061495) and three variants in the upstream *COL27A1* gene (rs2567706, rs2241671 and rs2567705) which were genotyped in both Achilles tendinopathy group and anterior cruciate ligament group. *TNC* gene inferred haplotype was too associated with Achilles tendinopathy risk [28].

Inelastic structure of tendons allows the resistance to very high forces. Capacity to withstand heavy loads before failure depends on the cross-sectional area and length, but excessive loading and tensile strains will in the end often result in tendinopathies. Extrinsic factors include overuse linked to sports activities, errors in training programme, faulty equipment and even weather conditions, such as sport activities performed in cold weather are also accounted as risk factors. Use of the Fluroquinolone based antibiotics have been proven to impact tendinopathies [36]. On the other hand, intrinsic factors will take under consideration several pathological conditions, for example association between Achilles tendinopathy and obesity/weight, genetic aspects as well as age, gender and height related factors of tendinopathy [37].

Professional athletes will have the more significant risk of developing tendinopathies mainly due to overuse by being involved in high-performance sports, with the additional negative effect for those being exposed to wide range of temperatures in sports that take place outdoors. Tendinopathy is increasing in prevalence in professional athletes, as well as in recreational athletes, accounting for substantial part of all sports injuries. Mostly affected are Achilles and patellar tendon, rotator cuff and extensor carpi radialis brevis, commonly known as tennis elbow tendon [38,39].

While tendinopathies are more manageable, overuse can lead to ruptures. Achilles tendon ruptures ended careers of up to 30% NBA basketball players, with their return to sports being as low as 61%. Athletes of similar movements including sudden stops, fast changes in direction and explosive acceleration are all putting an increased stress on lower body tendon complexes. Once injury occurs, most of the athletes will still find a way to return to the competitive sports, but their careers will be shorter and their performance will be decreased compared to their previous baseline [40,41]. In 2015. Goodlin *at al.* performed an interesting pilot program on fourteen triathletes, where they were genotyped and educated about their genetic make-up and personal risk profile. Participants responded positive, found it informative and it was reported that most acted upon their genetic results [42].

5. Conclusions

For professional, as well as for recreational athletes, knowledge of their genetic risk factors could prove to be useful, as it contributes to risk of soft tissue injuries. Additional knowledge of risk status could be used in modifying extrinsic factors and taking pre-emptive actions through more thorough conditional training, by incorporating more resting periods paired with preventive exercises to reduce risk of injury occurrence. Genetics of sports injuries is still very limited mainly in size of the cohorts that are being investigated, so every piece of additional genetic results of different populations adds to the bigger picture.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Medical ethics committee of the School of Medicine, University of Zagreb (380-59-10106-18-111/100, 24.05.2018.) and by the Ethics committee of the Faculty of Kinesiology, University of Zagreb (27.04.2016.).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study is available upon request to the corresponding author. Data was not made publicly available to maintain patient privacy.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

COL	collagen
SNP	single nucleotide polymorphisms
TNC	tenascin C
TEN	tendinopathy
DNA	deoxyribonucleic acid
PCR	polymerase chain reaction
FDR	false discovery rate
CON	control group
BMI	body mass index
HWE	Hardy-Weinberg equilibrium
MMP 3	matrix metaloproteinase-3
ESRRB	estrogen-related receptor beta
MiRNA	micro ribonucleic acid

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