The Apoptosis Pathway and the Genetic Predisposition to Achilles Tendinopathy

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ABSTRACT: Achilles tendinopathy (AT) is a degenerative condition for which several risk factors have been implicated including components of the inflammatory pathway. The aim was to assess functional variants within genes encoding components of the apoptosis signaling cascade and the effectiveness of a polygenic apoptosis profile to capture tendinopathy (TEN) risk. A total of 358 unaffected control (CON) participants [159 South Africa (SA CON) and 199 Australia (AUS CON)] and 166 affected AT (TEN) participants (87 SA TEN and 79 AUS TEN) were genotyped for four variants [*CASP8* (rs384129), *CASP8* (rs1045485), *NOS3* (rs1799983), and *NOS2* (rs2779249)]. Logistic regression was used to derive risk models for AT. A receiver operator characteristic (ROC) curve was plotted to determine the effectiveness of a model to capture AT risk. This study indicates the independent association of *CASP8*_rs1045485 and *CASP8*_rs3834129 as well as their haplotype with AT risk and the identification of an optimal model which included genetic loci *CASP8*_rs384129 and *CASP8*_rs1045485 together with sex to capture AT risk in both SA and AUS. Collectively, these results further implicate the apoptosis signaling cascade as one of the biological pathways involved in the development of AT. J Orthop Res 30:1719–1724, 2012

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Mechanical overload is an extrinsic risk factor for the development of Achilles tendinopathy (AT). Genetic risk factors[1–5] have recently been explored to explain the inter-individual variation in susceptibility to chronic AT in response to mechanical overload and to identify the biologically significant pathways underlying tendinopathy.

The normal tendon healing process, in response to overload, involves the removal of damaged tenocytes by cytokine-mediated apoptosis. Repetitive loading may, however, change the extracellular matrix (ECM) composition and result in excessive tenocyte apoptosis.[6,7] Tenocytes are required to maintain homeostasis of the ECM by regulating the balance between ECM synthesis and degradation,[8] therefore excessive tenocyte apoptosis which has been observed in tendinopathy,[9] may compromise the ability of the tendon to regulate repair processes. Identification of the biological mechanisms driving abnormal apoptosis are therefore of interest in understanding the pathophysiology of tendinopathy.

The caspase and nitric oxide pathways play a role in tendon apoptosis and were explored.[10,11] The expression of caspase-8 (*CASP8)* was found to be elevated in tendinopathy.[10] The enzyme that catalyses the formation NO is nitric oxide synthase (NOS) and both iNOS and eNOS were found to be elevated in tendinopathy.[12]

The aim of this study was to investigate polymorphisms within several candidate genes in the inflammatory pathway leading to apoptosis for associations to increased risk of AT. This study also aimed to assess the effectiveness of a genetic risk model for a single signaling cascade, the apoptosis cascade, to predict risk of developing AT, using a polygenic profile.

METHODS

Participants

The South African (SA) and Australian (AUS) participants were of self-reported European Caucasian ancestry. A total of 159 asymptomatic control participants (SA CON) and 87 with diagnosed Achilles tendinopathy (SA TEN) together with 199 AUS CON and 79 AUS TEN participants were recruited for the study as previously described.[1,2,5,13,14] Participants signed informed consent forms according to the Declaration of Helsinki, provided personal particulars and completed a questionnaire regarding medical history.[13] Approval for the study was obtained from the Human Research Ethics Committee of the Faculty of Health Sciences, The University of Cape Town and Human Ethics Committee of La Trobe and Deakin Universities, Melbourne, Australia.

DNA Extraction

DNA was extracted for all participants[1,14] as previously described.

Genotyping

Four functional polymorphisms previously associated with multifactorial phenotypes were investigated: *CASP8*,[15] rs3834129 and rs1045485, *NOS3* [16] rs1799983, and *NOS2* [17] rs2779249 (Supplement A Figs. 1–3). Genotyping of

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CASP8_rs384129, CASP8_rs1045485, and NOS2_rs2779249 was conducted using the Taqman method and NOS3_rs1799983 was genotyped using restriction fragment length polymorphism analysis (RFLP; Supplement A). All PCR reactions were performed on a thermal cycler (Hybaid; PCR Express, Middlesex, UK). For the Taqman assays, fluorescence was measured in each sample well after PCR using the 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). The data generated were analyzed with SDS2.3 software (Applied Biosystems). For quality control purposes, several negative controls (no DNA) were always included together with at least the same five repeat samples on every PCR plate for both RFLP and Taqman analysis.

Statistics

Basic characteristics of the study groups were presented and summarized previously [1,2,5,13,14,17]. The relationship between the genotypes and AT susceptibility was tested and found not to differ significantly between the countries. The data from the population groups were combined for all further analyses. Age, sex, country and whether the individual was born in the specific country were considered confounders and were adjusted for in all analyses by including them in the models as fixed effects. Logistic regression was used to compare the TEN and CON groups, as well as the countries with respect to genotype, allele and allele-combination frequencies. Significant genotype associations were further examined to determine whether it was the result of heterozygote, recessive or a dominant effect, by recoding the genotypes appropriately in the logistic regression models. Haplotype and allele combination associations were tested for additive, dominant and recessive models on the logit scale.

Inflammatory Risk Model for AT

Logistic regression was used to derive risk models for AT. Three models were constructed; the first incorporated the four known confounders and the genotypes at the four loci implicated in the apoptosis signaling cascade (CASP8_rs384129, CASP8_rs1045485, NOS3_rs1799983, NOS2_rs2779249).[5] The second contained the same factors as the first, plus the interleukin loci previously genotyped (IL-6_rs1800795; IL1β_rs16944; IL1β_rs1143627). The optimal model was backwards selected from the first, using Akaike criterion.

A receiver operating characteristic (ROC) curve[18] was constructed for each of the three logistic regression models to compare the effectiveness of each model to predict TEN risk. The area under the ROC curve (AUC) was used to quantify the overall ability of the model to discriminate between diagnostic groups based on genotype risk.

Results corresponding to a p-value of <0.05 were described as significant. The programming environment, R[19] and R packages were used for all analyses. The R package, genetics[20] was used to estimate genotype and allele frequencies and Hardy–Weinberg (HWE) equilibrium probabilities. Frequencies of allele combinations were inferred and analyzed using the R package, haplo.stats.[21,22] ROC curves were created using the R package Epi.[23]

RESULTS

Genotype and Allele Frequency Distributions

Genotype and minor allele frequency distributions for each of the polymorphisms together with the HWE p-values are shown in Table I.

CASP8_rs3834129

A significant difference in the genotype ($p = 0.0294$) but not the allelic distribution ($p = 0.2528$) was detected for CASP8_rs3834129 between the CON and TEN groups, after adjusting for the confounders. A heterozygote advantage model provided the best fit (OR = 0.61; $p = 0.0141$; 95% CI: 0.40–0.90); the odds of TEN with D/I (deletion allele/insertion allele) is 39% less than the odds with either (I/I or D/D) homozygote. A dominant model for the minor allele (I) also provided a significant fit (OR = 0.60; $p = 0.0215$; 95% CI: 0.38–0.93); with a D/D genotype the odds of TEN is 67% more (OR = 1.67; CI 1.08–2.60) than the D/I or I/I genotypes. The distribution for rs3834129 was similar between SA and AUS ($p = 0.860$ and $p = 0.3578$) after adjusting for the confounders.

CASP8_rs1045485

Significant differences in the genotype ($p = 0.0009$) and allele ($p = 0.00027$) distributions were detected for CASP8_rs1045485 between the two countries after adjusting for the confounders. Significant differences were detected in the genotype ($p = 0.0213$) and allele ($p = 0.0097$) distributions between the CON and TEN groups, after adjusting for the confounders. The significant allelic effect can be interpreted as: Each C allele reduces the odds of TEN by 41% (OR = 0.59; 95% CI: 0.39–0.87). Investigating the significant genotype effect showed two transmission models with significant fits, the heterozygote advantage (only the G/C genotype) reduces the odds of TEN (OR = 0.56; $p = 0.0094$; 95% CI: 0.35–0.86) compared to both homozygotes; and the dominant model (any minor C allele) either C/C or C/G genotype reduces the odds of TEN (OR = 0.55; $p = 0.0065$; 95% CI: 0.35–0.84) compared to G/G.

NOS3_rs1799983

No significant differences in the genotype or allele distributions were detected for NOs3_rs1799983.

NOS2_rs2779249

No significant differences in the genotype ($p = 0.3443$) or allele ($p = 0.6655$) distributions were detected for NOS2_rs2779249 between the CON and TEN groups. The genotype ($p = 0.0362$) and allele ($p = 0.0127$) distributions were, however, found to be significantly different between SA and AUS after adjusting for the confounders.

Interactions: Allele Combinations

Frequencies were inferred for the allele combinations CASP8_rs384129, CASP8_rs1045485, NOS3_rs1799983, and NOS2_rs2779249. The most common allele combinations were D-G-G-C (CON = 17%; TEN = 21%) and I-G-G-C (CON = 18%; TEN 16%); whereas both I-C-T-A and I-C-T-C were detected at frequencies below 1% in CON. The four-way allelic combination was not significantly associated with AT susceptibility after adjusting for the confounders and similarly nor were any of the three-way allelic combinations.

Table I. Genotype Frequency Distributions and Minor Allele Frequencies of *CASP8*_rs3834129, *CASP8*_rs1045485, *NOS3*_rs1799983, and N*OS2*_rs2779249 Polymorphisms in Control (CON) and Achilles Tendinopathy (TEN) Groups of South Africa (SA) and Australia (AUS)

	CON		TEN		*P*-values	
	SA	AUS	SA	AUS	Country	Diagnosis
CASP8_rs3834129						
N	156	189	82	76		
D/D	0.26	0.21	0.32	0.34	0.0860	**0.0294**
D/I	0.48	0.60	0.41	0.45		
I/I	0.26	0.20	0.27	0.21		
I	0.50	0.49	0.48	0.43	0.3578	0.2528
HWE	0.6330	**0.0089**	0.1261	0.4843		
CASP8_rs1045485						
N	159	199	84	79		
G/G	0.70	0.54	0.80	0.67	**0.0009**	**0.0213**
G/C	0.26	0.44	0.19	0.30		
C/C	0.03	0.02	0.01	0.03		
C	0.16	0.24	0.11	0.18	**0.0027**	**0.0097**
HWE	0.5729	**0.0032**	1.0000	1.0000		
NOS3_rs1799983						
N	151	174	87	76		
G/G	0.42	0.41	0.39	0.42	0.6437	0.3907
G/T	0.48	0.51	0.54	0.53		
T/T	0.11	0.09	0.07	0.05		
T	0.34	0.34	0.34	0.32	0.7495	0.6755
HWE	0.5896	0.1269	0.0920	0.1078		
NOS2_rs2779249						
N	154	191	82	78		
C/C	0.51	0.38	0.49	0.47	**0.0362**	0.3443
C/A	0.42	0.55	0.40	0.38		
A/A	0.07	0.07	0.11	0.14		
A	0.28	0.35	0.31	0.33	**0.0127**	0.6655
HWE	0.6941	**0.0041**	0.6081	0.3057		

p-Values are for the difference between countries and between diagnostic groups, respectively, adjusted for each other, age, sex and whether or not a person was investigated in his/her country of birth. HWE are exact *p*-values from tests of Hardy–Weinberg equilibrium. The genotype *p*-value is from a 2 degree of freedom test, with genotypes as categories. The allelic *p*-value is from additive allelic model on logit scale. *N* is number of samples genotyped. Bold *p*-values are <0.05.

The *CASP8* inferred haplotype was significantly associated with AT risk for additive ($p = 0.0210$), dominant ($p = 0.0052$), and recessive ($p = 0.0036$) allelic combination models. The D-C inferred allele combination was present in 15% of CON and 9% of TEN and showed a dominant protective effect such that an individual needs only one of those combinations to be protected against AT. While the D-G inferred allele combination was present in 35% of CON and 45% of TEN; showing a recessive risk effect such that you need to be homozygous for D-G allele combination to be at increased risk of AT.

Inflammatory Risk Models for Achilles Tendinopathy

Figure 1A shows a ROC curve of the model containing the four known confounders and the genotype data from CASP8_rs384129, CASP8_rs1045485, NOS3_rs1799983, and NOS2_rs2779249 to predict AT risk; AUC = 0.684 (132 TEN; 281 CON); and sensitivity = 62.9%, and specificity = 66.2%.

The model which contained genotypes for IL6_rs1800795, IL1β_rs16944, IL1β_rs1143627, CASP8_rs384129, CASP8_rs1045485, NOS3_rs1799983, and NOS2_rs2779249) and the confounders had an AUC = 0.705 (244 CON and 116 TEN); sensitivity = 45.7 and specificity = 84%.

The factors which jointly contributed to the optimal model for evaluating risk assessment of AT were the genetic loci CASP8_rs384129 and CASP8_rs1045485 and sex (Table II); the AUC = 0.667 (151 TEN and 336 CON; Fig. 1B) sensitivity = 60.9% and specificity = 64.3%.

DISCUSSION

The main findings are the association of the *CASP8* polymorphisms and their haplotype, and the

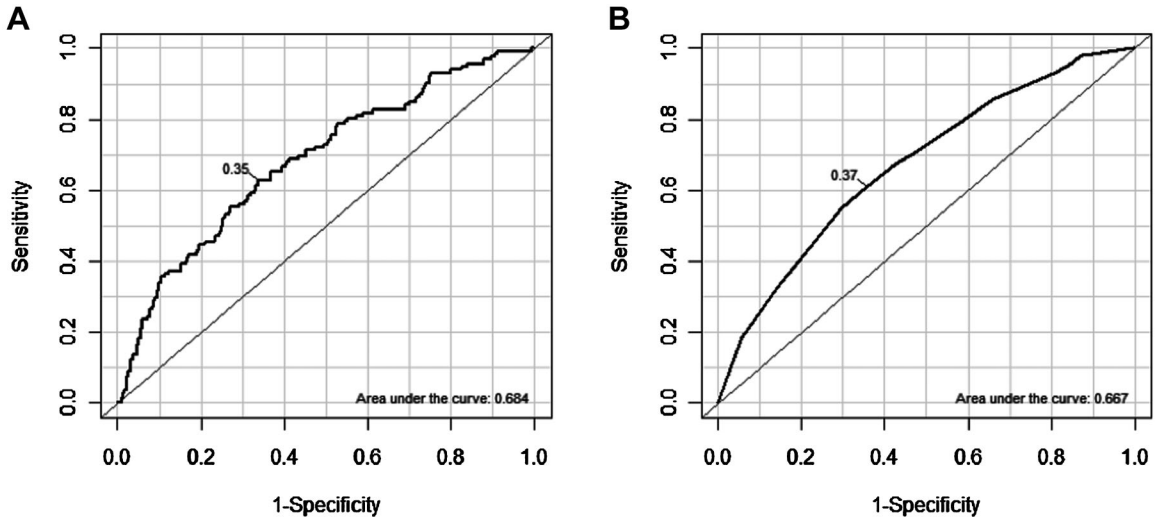

Figure 1. Receiver operating characteristic (ROC) curve of the apoptosis cascade profile (bold curve) to determine the true positive (sensitivity) versus true negative (specificity) rate for various cut-offs in determining risk of Achilles tendinopathy. The straight line indicates where sensitivity = 1-specificity and AUC = 0.5. The optimal cut-off which yields the maximum sensitivity plus specificity is indicated on the graph with an arrow. A: The logistic regression model containing the confounders sex, age, country, and born-here and the genotype data from CASP8_rs384129, CASP8_rs1045485, NOS3_rs1799983, NOS2_rs2779249) to predict AT risk; AUC = 0.684; sensitivity = 62.9% and specificity = 66.2%. CON = 159; TEN = 93. B: The "optimal" model, summarized in Table II, containing sex and genotype data from CASP8_rs384129 and CASP8_rs1045485. AUC = 0.667; sensitivity = 60.9% and specificity = 64.3%. CON = 336; TEN = 151.

identification of an apoptosis polygenic profile to capture the risk of AT.

The recessive model for CASP8_rs3834129 suggests that individuals with a D/D genotype have a 68% higher risk of AT than those with either I/I or D/I genotypes. This finding is unexpected since the del allele destroys a Sp1-binding element which results in decreased caspase-8 expression.[24] Reduced caspase-8 expression should protect against excessive apoptosis and the deletion allele should therefore protect against AT. Perhaps Sp1 can have different effects under different transcriptional control in different cell types, as Sp1 can both repress and activate transcription.[25] Future studies should explore the effect of this polymorphism in tenocytes to aid the understanding of this association. An alternative hypothesis may include that caspase expression within the inflammatory cells infiltrating the tendon tissue in response to microtrauma is reduced and the effect the inflammatory cells in the tendon is prolonged leading to an increase in apoptosis. Interestingly, the heterozygote advantage model predicts that if one is heterozygous, D/I genotype, at this locus there is a reduced risk compared to either homozygote (D/D and I/I).

The wild-type Asp amino acid at the CASP8_rs1045485 locus is predicted to be the more stable form of caspase-8[26] and thus the association of the C allele or genotypes containing the C allele with reduced AT risks makes biological sense. There was a significant protective effect of the heterozygote genotype, C/G compared to G/G genotypes. The C/C genotype was rare (2%) so it was not surprising that a heterozygote (G/C reduces AT risk by 44% compared to G/G + C/C) and an additive allelic (each C allele

Table II. Summary of the "Optimal" Logistic Regression Model Used for ROC Analysis

Factor in model	Level	Coefficient	SE	*p*-Value
Sex	Female	0		
	Male	0.967	0.216	0.001
*CASP8*_rs1045485	G/G	0		
*CASP8*_rs1045485	G/C	−0.769	0.236	0.001
*CASP8*_rs1045485	C/C	−1.304	1.102	0.237
*CASP8*_rs3834129	D/D	0		
*CASP8*_rs3834129	D/I	−0.659	0.241	0.006
*CASP8*_rs3834129	I/I	−0.435	0.283	0.124

The coefficients are used to predict at risk, which is used to calculate points on the ROC curve. *p*-values are from joint model, so adjusted for each other, all assessing the effect of specific factor level compared to reference level—the absent one (female; G/G and D/D, respectively).

reduces risk by 41%) and a dominant model (C/C + C/G reduces AT risk by 45% compared to G/G) all provided significant fits.

The *CASP8* polymorphism associations were mirrored in the *CASP8* haplotype. The *CASP8* D-C haplotype was associated with reduced AT risk in the additive, dominant and recessive allelic combination models. Genotyping other SNPs in the region implicated by the haplotype may provide more informative haplotypes in identifying the critical casual region.

No associations with AT were observed at the *NOS3* or *NOS2* loci; however, they still remain good candidate genes.

An interesting finding was the heterozygote advantage model noted at the *CASP8* loci. In a preliminary exploratory analysis, a heterozygous advantage model was also noted at the *NOS2* locus in the AUS group ($p = 0.049$; results not shown). One hypothesis suggests a "*Goldilocks effect*" within the apoptosis pathway, both too much and too little expression of apoptotic mediators may be detrimental and lead to pathology. To date, reported risk or protection of AT have only been associated with homozygous genotypes; heterozygous protection has not been observed in the genetic predisposition to injury. This phenomenon is not uncommon in susceptibility to infectious disease.[27] Several models with significant fits were identified for the two loci within *CASP8*. For this reason, investigations on larger study groups are required before a firm conclusion can be reached.

Lastly, this study suggests that the more biomarkers incorporated into the design of a risk profile, the greater the effectiveness to predict risk, as would be expected in a polygenic condition (AUC = 0.705). The optimal risk model determined from the variants tested in this study suggests that the two loci *CASP8* together with sex is sufficient to predict AT risk (AUC = 0.667). The optimal model estimates that the minimum risk for AT occurs in females who are homozygous C/C and heterozygous D/I for CASP8_rs384129 and CASP8_rs1045485, respectively, and on the contrary males with the G/G and D/D genotypes at the two *CASP8* loci are at maximum risk for AT. Although all inferred allele combinations were not significantly associated with AT risk, the ROC analysis suggests that the loci are collectively able to discriminate between affected and unaffected individuals. This suggests that the cumulative effect of these protein products contribute to AT risk.

Increased synthesis of cytokines and growth factors could potentially affect the metabolism of proteoglycans which can eventually lead to the compromised ECM observed in chronic AT.[28] The elucidation of the cumulative biological significance of variants within genes encoding these signaling molecules on tendon metabolism and cellularity needs to be explored.

Collectively, these results further implicate the apoptosis signaling cascade as one of the biological pathways involved in the development of AT. The associations observed in this study should be explored in larger independent groups, of different ethnicities, to elucidate the biological significance of the apoptosis signaling cascade in musculoskeletal soft tissue injuries.

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