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Research paper

Variation within three apoptosis associated genes as potential risk factors for Achilles tendinopathy in a British based case–control cohort

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ABSTRACT

Achilles tendon pathology (ATP) is a degenerative condition which exhibits excessive tenocyte apoptosis. Tumour necrosis factor receptor 1 (TNFR1), caspase-3 (CASP3) and caspase-8 (CASP8) are important regulators of apoptosis. To date, the effects of variation within the genes for TNFR1 and CASP3 as risk factors for ATP have not been described. There is evidence that two single nucleotide polymorphisms (SNPs) within the *CASP8* gene are associated with ATP, but only in populations from the Southern Hemisphere. The primary aim of this study was to determine whether SNPs within the *TNFRSF1A* and *CASP3* genes were associated with ATP in British Caucasians. We additionally sought to determine whether copy number variation (CNV) within the *CASP8* gene was associated with ATP. We recruited 262 (131 ATP cases and 131 asymptomatic controls) Caucasian participants for this genetic association study and used quantitative PCR with chi-squared (χ^2) tests and ANOVA to detect significant associations. For our entire cohort, we found no association between the *TNFRSF1A* rs4149577 ($p = 0.561$), *CASP3* rs1049253 ($p = 0.643$) and *CASP8* variants ($p = 0.219$) and ATP. Likewise, when we tested potential interactions between gender, genotype and the risk of ATP, we found no association with the variants investigated. In conclusion, the *TNFRSF1A*, *CASP3* and *CASP8* gene variants were not associated with ATP in British Caucasians.

1. Introduction

The Achilles tendon is prone to damage and rupture (Lesic and Bumbasirevic, 2004). Increased stress on tendons during exercise can cause such damage in both professional and recreational athletes, typically as a result of repetitive mechanical loading (Maffulli et al., 2003; Xu and Murrell, 2008; Collins and Raleigh, 2009). Achilles tendon pathology (ATP) can manifest as either insertional or non-insertional pathologies (Lesic and Bumbasirevic, 2004; Schepsis et al., 2002). Alternatively the pathology can present as partial or complete rupture (Lesic and Bumbasirevic, 2004; Schepsis et al., 2002) and lead to long-term incapacitation and a reduction in physical activity (Lesic and Bumbasirevic, 2004).

A range of factors, including genetics, have been shown to increase the risk of ATP (Collins and Raleigh, 2009; El Khoury et al., 2013; Meeuwisse, 1994; Raleigh and Collins, 2012). For example, genes that encode proteins with a role in maintaining the integrity of the tendon extracellular matrix (ECM) have been shown to associate with ATP (Collins and Raleigh, 2009; El Khoury et al., 2013; Raleigh and Collins, 2012). Apoptosis is a normal mechanism in tendon healing to remove damaged tenocytes (September et al., 2012), however, relatively little is known about genetic variation within genes involved in apoptosis and the risk of ATP. Previous work in this area has been limited to variants within the *CASP8* (rs384129, rs1045485), *NOS2* (rs2779249) and *NOS3* (rs1799983) genes in two cohorts from the Southern Hemisphere (South Africa and Australia) (Nell et al., 2012).

The *TNFRSF1A* gene encodes tumour necrosis factor receptor 1 (TNFR1), a cell receptor that can signal apoptosis in response to the pro-inflammatory cytokine tumour necrosis factor-alpha (TNF-α) (Hosaka et al., 2005; Gaida et al., 2012). TNFR1 mRNA and protein have recently been identified in human Achilles tendon (Gaida et al., 2012) and in cultured tenocytes (Backman et al., 2013). Importantly, TNFR1 is known to be highly expressed in tenocytes isolated from Achilles tendinosis (Gaida et al., 2012) but the functional or pathologic significance of this is not clear. Single nucleotide polymorphisms (SNPs) within the *TNFRSF1A* gene, especially the rs4149577 variant, have previously been associated with other musculoskeletal (Karaderi et al., 2012) and inflammatory diseases (Park et al., 2013). However, the role of this variant as a risk factor for ATP has not been investigated.

Caspases form a family of proteases that are important in the regulation of apoptosis (Hengartner, 2000). Caspase-3 can selectively cleave target proteins after aspartate residues in their primary sequence (Hengartner, 2000; Kirsch et al., 1999). Although apoptosis can be activated through a number of complex pathways, caspase-3 appears to

Abbreviations: ANOVA, analysis of variance; ATP, Achilles tendon pathology; CASP, caspases; CN, copy number; CNV, Copy number variant; CON, control; NOS, nitric oxide synthase; SNP, Single nucleotide polymorphism; TNFRSF, tumour necrosis factor receptor superfamily; UTR, untranslated region.

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have a critical role in chromatin condensation and DNA fragmentation (Porter and Jänicke, 1999). The *CASP3* rs1049253 variant resides within the 3′ untranslated region (UTR) of the gene and can influence the binding of miR-885-5p to *CASP3* mRNA (Guan et al., 2013). Guan et al. (2013) showed that the CC genotype of this variant influences levels of *CASP3* mRNA expression (Guan et al., 2013). Like the *TNFRSF1A* gene, the role of the *CASP3* rs1049253 variant in predisposing to ATP is unknown.

Another important regulator of apoptosis is caspase-8. Caspase-8, encoded by the *CASP8* gene, can activate downstream effector caspases (Hosaka et al., 2005). It is known to regulate apoptosis of tendon fibroblasts (September et al., 2012). Regulating the balance between synthesis and degradation is essential in maintaining ECM homeostasis and the removal of damaged tendon fibroblasts during normal tendon turnover is important (September et al., 2012). However, atypical tenocyte apoptosis has been shown in tendinopathy, with elevated expression levels of *CASP8* observed (Nell et al., 2012). Two SNPs within the *CASP8* gene have been associated with ATP (Nell et al., 2012) but the role of larger scale variation within this gene as a risk factor has not been considered.

Although our understanding of the role that SNPs play in ATP is growing (Collins and Raleigh, 2009; El Khoury et al., 2013; Raleigh and Collins, 2012; Nell et al., 2012), there has yet to be an investigation into the influence of copy number variation (CNV) as a predisposing factor. Copy number variants (CNVs) are segments of DNA greater than 1 kb in size, which show altered copy number (CN) when compared with a reference genome (Redon et al., 2006). CNVs can influence phenotypes by altering gene dosage and disrupting coding sequences of DNA (Yang et al., 2008). Indeed, predisposition to certain diseases appears to be associated with CNV (Yang et al., 2008; Estivill and Armengol, 2007). The *CASP8* gene is known to harbour a CN variant that spans intron 11–intron 12 of the nucleotide sequence (as reported in the Database of Genomic Variants (DGV) (http://projects.tcag.ca/variation/)). With regard to the role of caspase 8 in apoptosis, and as SNPs within this gene have been associated with ATP (Nell et al., 2012), we considered that CNV within *CASP8* might predispose to ATP. Additionally, for the reasons outlined in the preceding paragraphs, we decided to investigate whether the *TNFRSF1A* rs4149577 and *CASP3* rs1049253 variants were additional risk factors for this pathology.

2. Methods

One hundred and thirty one British Caucasian participants diagnosed with ATP and 131 asymptomatic British Caucasian controls (CON) were recruited for this genetic association study. ATP participants were recruited through The County Clinic in Northampton, UK. Participants within the CON group (physically active individuals without any history of ATP) were recruited from the East Midlands region of the UK. Cases of Achilles tendinopathy typically presented with gradual progressive pain with early-morning pain/stiffness in the Achilles tendon area. Affected individuals were diagnosed by the clinical author (WJR) using established, published criteria (Schepsis et al., 2002; Kader et al., 2002; Mokone et al., 2006). Diagnosis of tendinopathy was objectively confirmed by imaging in all cases by either MRI and/or ultrasound of the affected Achilles tendon. The ATP group consisted of participants with non-insertional (N = 47) or insertional (N = 29) tendinopathy along with those presenting with partial or complete Achilles tendon rupture (N = 23). We further recruited an additional 29 cases with more than one type of pathology (insertional, non-insertional and rupture). Three individuals originally recruited as controls later reported symptoms of ATP but the specific type of tendinopathy for these was not obtained. However, we conducted our main analysis by including these three individuals as cases. We also conducted a separate analysis where they were excluded. Subsequent to initial diagnosis, 15 cases with Achilles tendinopathy went on to develop ruptures. As each subpathology (insertional, non-insertional and rupture) was relatively small in number we pooled all cases as having the combined condition of Achilles tendon pathology and this was the main phenotype we chose to investigate. Additional association analysis on each sub-pathology was, where appropriate, investigated but this was statistically underpowered due to the small sample sizes. All participants completed a physical activity/medical history/injury questionnaire and gave written, informed consent. The study was approved by the Research Ethics Committee of the University of Northampton, United Kingdom.

DNA was extracted from 2 mL of saliva collected using ORAGENE-DNA kits (OG-500) and DNA purification was carried out using the prepIT-L2P DNA extraction kit (DNA Genotek Inc., Ontario, Canada). Quantitative PCR (qPCR) reactions were run on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, California, USA) in 96-well plates, using fluorescence-based TaqMan assays. The genotyping assays for the *TNFRSF1A* rs4149577, *CASP3* rs1049253 and *CASP8* variants were selected from Applied Biosystems. PCR reactions contained probes and primers in a mastermix containing AmpliTaq DNA Polymerase Gold and 10 ng of DNA. The cycling conditions consisted of a holding phase at 95 °C for 10 min, followed by 40 cycles of denaturing at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. Quality control for our SNP genotyping consisted of running both within and between plate replicates to assess for any errors in genotype determination. We also included no template controls (NTCs) within each qPCR run. For the copy number assay, each sample was repeated in quadruplicate and included NTCs.

For the *TNFRSF1A* rs4149577 and *CASP3* rs1049253 SNPs, the TaqMan Genotyping Assays (C_2645708_10 and C_11683739_10 respectively) contained both FAM and VIC reporter dye labelled probes to discriminate between genotypes, along with ROX dye as the passive reference. Genotypes were called using StepOne Software version 2.1 (Applied Biosystems, Foster City, California, USA). CNV spanning intron 11–intron 12 of the *CASP8* gene was determined using the TaqMan copy number assay Hs02601709_cn along with the reference assay for RNase P. QPCR was performed as a duplex reaction and all CN data were exported into CopyCaller Software version 2.0 (Applied Biosystems, Foster City, California, USA) for calculation of discrete and continuous CN. CN of the target gene was normalised relative to the reference RNase P gene. We used CopyCaller Software to calculate confidence and z-score quality metrics. CN calls were inspected for reproducibility and accepted with a z-score of <1.75. To establish PCR efficiency of both assays (*CASP8* and RNase P), qPCR was carried out using serial dilutions of genomic DNA. Efficiency (E) was calculated using the equation $E = 10^{(-1/m)} - 1$ (Liu et al., 2011).

Data were analysed using IBM SPSS Statistics version 20 (IBM Corp. Armonk, NY). One-way analysis of variance (ANOVA) was used to determine any significant differences between the characteristics (age, height, weight, BMI) of the ATP and CON groups. A Pearson's chi-squared (χ^2) test was used to determine any significant differences between the gender of the ATP and CON groups. A χ^2 or Fisher's exact test was used to analyse for differences in genotype and allele frequency distributions for the *TNFRSF1A* rs4149577 and *CASP3* rs1049253 variants, as well as differences in discrete CN (<2, =2, >2 copies) for the *CASP8* gene in the ATP and CON groups. Differences in continuous CN values between ATP and CON groups were analysed using a non-parametric Mann Whitney *U*-test. Data were also analysed by gender and, where appropriate, comparisons were also made between the ATP sub-pathologies and CON groups. In all analyses, significance was accepted at $p < 0.05$. Hardy–Weinberg equilibrium (HWE) was established using a HWE calculator (Michael H. Court, 2005–2008) accessed from www.tufts.edu/~mcourt01/Documents/Court lab — HW calculator.xls. $p < 0.05$ was considered to be a deviation from HWE. The statistical power of our SNP analysis was calculated using Quanto version 1.2 (http://hydra.usc.edu/gxe) (Gauderman, 2002). Assuming an odds ratio (OR) of 2.2 and a recessive mode of inheritance, our analysis had 80% power to detect associations at the $p < 0.05$ significance level. For our CNV analysis we used sampsize (Version 0.6), available

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