Components of the transforming growth factor-β family and the pathogenesis of human Achilles tendon pathology—a genetic association study

Michael Posthumus\(^1\), Malcolm Collins\(^2,3\), Jill Cook\(^4\), Christopher J. Handley\(^5\), William J. Ribbons\(^1\), Roger K. W. Smith\(^6\), Martin P. Schwellnus\(^2\) and Stuart M. Raleigh\(^1\)

See page 2005 for the editorial comment on this article (doi:10.1093/rheumatology/keq150)

Abstract

Objectives. Achilles tendon pathology is a multifactorial condition for which various risk factors, including genetic factors, have been identified. Gene transfection of two members of the TGF-β family, TGF-β1 and growth/differentiation factor-5 (GDF-5), have been shown to enhance tendon repair and mechanical strength within animal Achilles tendon injury models. The objective of this study was to investigate whether two functional 5’ untranslated region (UTR) single nucleotide polymorphisms (SNPs), the \(\text{TGFB1}\) rs1800469 variant and the \(\text{GDF5}\) rs143383 variant, were associated with Achilles tendon pathology (ATP) within an Australian (‘AUS’) and a South African (‘SA’) case–control cohort.

Methods. One hundred and seventy-one subjects (58 AUS and 112 SA) with ATP and 235 (142 AUS and 96 SA) asymptomatic control (CON) subjects were genotyped for the selected SNPs using custom-designed Taqman assays. A \(\chi^2\)-analysis or Fisher’s exact test was used to analyse any differences in the genotype and allele frequencies. Significance was accepted when \(P < 0.05\).

Results. There were no significant \(\text{TGFB1}\) rs1800469 genotype (\(P = 0.491\)) or allele (\(P = 0.400\)) frequency differences between the ATP and CON groups. The TT genotype of the \(\text{GDF5}\) rs143383 variant was significantly over-represented in the ATP group of the AUS cohort (\(P = 0.011\); odds ratio (OR) = 2.24; 95% CI 1.21, 4.16), and when the AUS and SA cohorts were combined (\(P = 0.004\); OR = 1.82; 95% CI 1.23, 2.74).

Conclusions. In conclusion, this study suggests that individuals with a TT genotype of the functional \(\text{GDF5}\) rs143383 variant have twice the risk of developing ATP. This finding highlights a role of GDF-5 in the pathogenesis of Achilles tendon pathology.

Key words: Growth/differentiation factor-5, Bone morphogenic protein 14, Cartilage-derived morphogenic protein 1, Gene, Tendinopathy, Tendon rupture, Tendon injury.

Introduction

Achilles tendon pathology, which includes chronic Achilles tendinopathy and acute Achilles tendon rupture, occurs as a consequence of acute or repetitive mechanical loading during occupational and sporting activities [1]. The exact mechanism and aetiology of these injuries are currently unknown; however, various intrinsic (including genetic factors) and extrinsic risk factors have been identified for both Achilles tendinopathy and Achilles tendon rupture [2]. Among the genetic risk factors, DNA sequence variants within genes that code for specific tendon
extracellular matrix proteins (COL5A1 [3, 4], TNC [5]) and those involved with their metabolism (MMP3 [6]) have recently been shown to be significantly associated with chronic Achilles tendinopathy. The TNC gene variant also appears to be associated with Achilles tendon rupture [5]. It has been proposed that these genetic factors may in the future be included in multifactorial models developed to identify individuals predisposed to Achilles tendon pathology [7, 8]. These models have significant clinical implication, such that individualized therapy and intervention may reduce the incidence of chronic Achilles tendon pathology and improve patient treatment outcomes. However, it is essential that further candidate genes be investigated as possible predisposing factors to Achilles tendon pathology.

The TGF-β1 superfamily, which includes various growth/differentiation factors (GDFs), plays an essential role in tissue (including tendon) growth and homeostasis. Two members of this family, TGF-β1 (an isofrom of TGF-β1) and GDF-5, have been shown to increase mechanical strength after gene transfection in experimentally injured animal Achilles tendons [9–11].

TGF-β1 is released in response to a number of stimuli (including mechanical loading [12]) and is known to increase cell proliferation, migration and the synthesis of extracellular matrix. The gene coding for TGF-β1, TGF1, is located on chromosome 19q13. The 5’ untranslated region (UTR) of the TGFβ1 gene contains a functional promoter single nucleotide polymorphism (SNP) (rs1800469, C/T) that has been associated with various multifactorial pathologies; including various cancers [13, 14], asthma [15, 16], chronic obstructive pulmonary disease [17], osteoporosis [18] and myocardial infarction [19]. In a study designed to determine the genetic contribution of circulating TGF-β1 concentrations, the TGFβ1 rs1800469 variant was significantly associated with TGF-β1 plasma concentration [20]. Moreover, retrospective regression analysis estimated the mean acid-activated TGF-β1 concentration to be approximately twice as high in the TT genotype compared with the CC genotype of the TGFβ1 rs1800469 variant [20]. Further investigation has attributed the change in TGF-β1 plasma levels to transcriptional suppression due to AP1 binding to the C allele of the TGFβ1 rs1800469 variant [17].

The specific role of GDF-5 [also known as bone morphogenetic protein-14 (BMP-14) or cartilage-derived morphogenetic protein-1 (CDMP-1)] in tendon is largely unknown [21]. GDF-5 is involved in the maintenance, development and repair of bones, cartilage and various other musculoskeletal soft tissues (including tendons) [21, 22]. The gene coding for GDF-5, GDF5, is located on chromosome 20q11. Mutations within the GDF5 gene are known to cause several inherited developmental disorders such as brachydactyly type C, Grebe and Hunter-Thompson forms of acromesomelic skeletal dysplasias and Du Pan syndrome [23–25]. A possible role of GDF-5 in tendon and ligament biology was first suggested by Wolfman et al. [26]. In this study, ectopic administration of GDF-5 resulted in neotendon formation. To further investigate the role of GDF-5 in tendon, Mikic et al. [27] examined the ultrastructural, compositional and mechanical characteristics of Achilles tendons from mutant GDF-5-deficient mice. The mutant GDF-5-deficient tendons were significantly weaker and contained ∼40% less collagen [27].

The 5’ UTR of the GDF5 gene contains a functional promoter SNP (rs143383; T/C) that has been associated with multifactorial disorders such as OA [28] and congenital hip dysplasia [29], as well as phenotypic data such as height, hip axis length and fracture risk [30]. The function of this SNP has been reported by luciferase reported assays [31] and differential allelic expression analysis [32, 33]. The T allele of the GDF5 rs143383 was correlated with reduced expression of the GDF5 gene within a wide range of soft tissues [32, 33].

The primary aim of this study was to determine whether the two selected functional polymorphisms (the GDF5 rs143383 variant and the TGFβ1 rs1800469 variant) were associated with Achilles tendon pathology within an Australian (AUS) and a South African (SA) case–control cohort. Due to the known functional effect of the chosen SNPs, we hypothesize that the CC genotype of the TGFβ1 rs1800469 variant and the TT genotype GDF5 rs143383 variant increase the risk of Achilles tendon pathology.

Methods

Subjects

The Australian case–control cohort. Fifty-nine Caucasian patients with documented Achilles tendon pathology, all of which were diagnosed with chronic Achilles tendinopathy (AUS TEN group), were recruited for this study by the Musculoskeletal Research Centre at La Trobe University in Melbourne, Australia, as previously described [4]. The diagnosis of chronic Achilles tendinopathy in all patients was made using clinical criteria, as previously described [3–5]. All diagnoses were confirmed with soft-tissue ultrasound examination of the affected Achilles tendon. An additional 142 apparently healthy unrelated Caucasian subjects without any history of asymptomatic Achilles tendonopathy (AUS TEN group) were recruited as controls for this study from Melbourne, Australia. The inclusion and exclusion criteria of the participants have been previously described [4].

The South African case–control cohort. In all, 112 Caucasian subjects diagnosed with Achilles tendon pathology (SA ATP group), including 73 with Achilles tendinopathy (SA TEN sub-group) and 39 with Achilles tendon ruptures (SA RUP sub-group) (complete ruptures, n = 36; partial ruptures, n = 3), were recruited for this study from the medical practice at the Sports Science Institute of South Africa and other clinical practices within the greater Cape Town area of South Africa, as previously described [3, 5]. Diagnosis of chronic Achilles tendinopathy in all patients was the same as described for the AUS cohort. Rupture of the Achilles tendon was confirmed
during surgery or by imaging. Ten of the subjects in the SA RUP sub-group had a history of Achilles tendinopathy. An additional 96 apparently healthy unrelated Caucasian subjects without any history of asymptomatic Achilles tendon injuries were recruited as controls (SA CON group) for this study from Cape Town, South Africa. The inclusion and exclusion criteria of the participants have been previously described [3, 5].

All volunteers in this study (AUS and SA cohorts) were required to complete an informed consent form according to the Declaration of Helsinki, and once recruited, subjects detailed personal particulars and medical history by means of a questionnaire. This study was approved by the Research Ethics Committee of the Faculty of Health Sciences within the University of Cape Town, South Africa, the Human Ethics Committee of La Trobe and Deakin Universities, Melbourne, Australia and the Research Ethics Committee of the University of Northampton, UK.

DNA extraction and SNP genotyping

For the AUS cohort, DNA was extracted from whole blood using a sequenced extraction technique (Flexigene DNA kit; Qiagen P/L, Valencia, CA, USA) as per the manufacturer’s recommendations. DNA for the SA cohort was extracted from whole blood using the procedure described by Lahiri and Nurnberger [34] and modified by Mokone et al. [3, 5].

All subjects were genotyped for the TGFβ1 rs1800469 variant and the GDF5 rs143383 variant using fluorescence-based Taqman assays (Applied Biosystems, Foster City, CA, USA). Custom-designed allele-specific probes and flanking primer sets (sequences available from the authors) were used along with a pre-made PCR mastermix containing AmpliTaq DNA Polymerase Gold (Applied Biosystems) in a final reaction volume of 8 μL. PCR consisted of a 10-min heat activation step (95°C) followed by 40 cycles of 15 s at 92°C and 1 min at 60°C. The PCR was performed on an Applied Biosystems StepOnePlus Real-Time PCR system (Applied Biosystems). Genotypes were automatically determined by the Applied Biosystems StepOnePlus Real-Time PCR software Version 2.1 (Applied Biosystems).

Statistical analyses

The required sample size for this study was determined using QUANTO Version 0.5 (http://hydra.usc.edu/gxe) [35]. Data were analysed using STATISTICA Version 8.0 (StatSoft, Tulsa, OK, USA) and Graphpad InStat Version 3 (Graphpad Software, San Diego, CA, USA) statistical programs. A one-way analysis of variance was used to determine any significant differences between the characteristics of the ATP and CON groups within the AUS and SA cohorts. A χ²-analysis or Fisher’s exact test was used to analyse any differences in the genotype and allele frequencies, as well as other categorical data between the groups. Significance was accepted when P < 0.05. Hardy–Weinberg equilibrium (HWE) was established using the program Genepop web version 3.4 (http://genepop.curtin.edu.au/).

Results

Subject characteristics

The AUS CON and AUS ATP groups were similarly matched for age at initial onset of injury, height and country of birth (Table 1). The AUS ATP group had a significantly greater frequency of males when compared with the AUS CON group. The AUS ATP group were recruited on average 8.8 (10.0) years after their initial symptoms. Once co-varied for age and gender, there were no further differences between the groups.

The SA ATP and SA CON groups were matched for country of birth, age at initial onset of injury, gender and height (Table 1). The SA ATP group was however significantly heavier with a significantly higher BMI.

Table 1 Characteristics of the AUS and SA ATP groups and their respective CON groups

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>AUS CON (n = 142)</th>
<th>AUS ATP (n = 59)</th>
<th>P-value</th>
<th>SA CON (n = 96)</th>
<th>SA ATP (n = 112)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>39.0 (12.1) (140)</td>
<td>40.3 (14.1) (59)</td>
<td>0.050</td>
<td>36.9 (9.9) (89)</td>
<td>40.2 (13.5) (107)</td>
<td>0.058</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>73.4 (13.8) (142)</td>
<td>80.4 (15.0) (59)</td>
<td>0.330</td>
<td>72.0 (12.3) (95)</td>
<td>80.8 (14.9) (106)</td>
<td>&lt;0.001b</td>
</tr>
<tr>
<td>Height, cm</td>
<td>171.7 (9.3) (141)</td>
<td>173.8 (9.5) (57)</td>
<td>0.150</td>
<td>175.4 (9.7) (93)</td>
<td>175.9 (8.7) (103)</td>
<td>0.706</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>24.9 (4.0) (141)</td>
<td>26.6 (4.1) (57)</td>
<td>0.517</td>
<td>23.3 (2.8) (93)</td>
<td>26.0 (3.9) (103)</td>
<td>&lt;0.001b</td>
</tr>
<tr>
<td>Gender, male, n (%)</td>
<td>142 (40.2)</td>
<td>59 (67.8)</td>
<td>&lt;0.001</td>
<td>95 (66.3)</td>
<td>111 (73.0)</td>
<td>0.299</td>
</tr>
<tr>
<td>Country of birth, Australia, n (%)</td>
<td>138 (84.8)</td>
<td>58 (75.9)</td>
<td>0.155</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Country of birth, South Africa, n (%)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>95 (75.8)</td>
<td>108 (75.0)</td>
<td>1.000</td>
</tr>
</tbody>
</table>

All values are expressed as mean (s.d.) (n) or when applicable as n (%). Significant values (P < 0.05) are shown in bold. aAge of the AUS chronic ATP groups (AUS ATP) and SA ATP subjects at the onset of symptoms. The subjects of the AUS ATP group were on average 49.18 (12.1) years old at the time of recruitment and 8.8 (10.0) years after their initial symptoms. The subjects of the SA ATP group were on average 48.2 (11.1) years old at the time of recruitment and 8.0 (9.0) years after their initial symptoms; bco-varied for gender and age at recruitment. ND: not determined.
There were however no genotype effects for the \textit{GDF5} rs143383 variant and the \textit{TGFB1} rs1800469 variant on any of the demographic or anthropometric subject characteristics within the SA and AUS cohorts (data not shown).

\textbf{Genotype and allele frequencies}

The genotype and allele frequency information for the \textit{TGFB1} rs1800469 variant and the \textit{GDF5} rs143383 variant are provided in Tables 2 and 3, respectively. There were no significant \textit{TGFB1} rs1800469 genotype or allele frequency differences between the ATP and CON groups within the SA cohort (Table 2). All groups of the \textit{TGFB1} rs1800469 variant were in HWE.

There were significant genotype differences between the AUS ATP and AUS CON groups for the \textit{GDF5} rs143383 variant ($P = 0.027$, Table 3). There were no significant \textit{GDF5} rs143383 allele frequency differences between the AUS CON and AUS ATP groups ($P = 0.070$). Although similar frequencies and trends were observed within the SA cohort, there were no significant genotype ($P = 0.189$) or allele ($P = 0.085$) differences. Similar genotype frequencies were observed between the SA RUP and TEN sub-groups. When the data from

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|c|c|}
\hline
\multicolumn{2}{|c|}{\textbf{GDF5 (rs143383) genotype}} & \multicolumn{2}{|c|}{\textbf{GDF5 (rs143383) allele}} & \multicolumn{3}{|c|}{\textbf{P-value}} & \multicolumn{2}{|c|}{\textbf{HWE}} \\
\hline
\textbf{n} & \textbf{TT} & \textbf{TC} & \textbf{CC} & \textbf{T} & \textbf{C} & \textbf{P-value} & \textbf{TEN} & \textbf{RUP} \\
\hline
\textbf{AUS cohort} &  &  &  &  &  &  &  &  \\
\textbf{ATP group} & 59 & 42.5 (31) & 33.9 (20) & 13.6 (8) & 69.5 (82) & 30.5 (36) & 0.027* &  \\
\textbf{CON groups} & 142 & 33.1 (47) & 52.8 (75) & 14.1 (20) & 59.5 (169) & 40.5 (115) & 0.296 &  \\
\textbf{SA cohort} & 205 &  &  &  &  &  &  &  \\
\textbf{ATP group} & 110 & 43.6 (48) & 41.8 (46) & 14.5 (16) & 64.5 (142) & 35.5 (78) & 0.085* & 0.408 \\
\textbf{CON groups} & 95 & 31.6 (30) & 48.4 (46) & 20.0 (19) & 55.8 (196) & 44.2 (64) & 0.837 &  \\
\textbf{TEN sub-group} & 73 & 42.5 (31) & 42.5 (31) & 15.1 (11) & 65.7 (93) & 36.3 (53) & 0.183* 0.462 &  \\
\textbf{RUP sub-group} & 37 & 46.0 (17) & 40.5 (15) & 13.5 (5) & 66.2 (49) & 33.8 (25) & 0.712 &  \\
\textbf{Combined cohorts} & 406 &  &  &  &  &  &  &  \\
\textbf{ATP groups} & 169 & 46.7 (79) & 39.1 (66) & 14.2 (24) & 66.3 (224) & 33.7 (114) & 0.013* & 0.199 \\
\textbf{CON groups} & 237 & 32.5 (77) & 51.1 (121) & 16.5 (39) & 58.0 (275) & 42.0 (199) &  &  \\
\hline
\end{tabular}
\caption{Genotype and allele frequency distribution of the functional SNP analysed within the 5’ UTR of the \textit{GDF5} (rs1800469) gene in the AUS ATP and AUS CON groups, as well as the SA CON and SA ATP groups, and the SA TEN and SA RUP sub-groups.}
\end{table}

Values are expressed as percentage of frequency with the number of subjects ($n$) in parentheses. HWE is the $P$-value for the exact tests for HWE. *ATP vs CON; #TEN vs RUP vs CON. Significant values ($P < 0.05$) are shown in bold.
the AUS and the SA cohort were combined, both genotype ($P = 0.013$) and allele ($P = 0.019$) frequencies were significantly different between the ATP and CON groups. All groups of the GDF5 rs143383 variant were in HWE.

Similar results were observed when individuals with a TT genotype for the GDF5 rs143383 variant were compared with individuals with a C allele (combined TC and CC; Fig. 1). Within the AUS cohort, the TT genotype was significantly over-represented within the ATP group ($P = 0.011$; odds ratio (OR) = 2.24; 95% CI 1.21, 4.16). Although there was a similar pattern in the SA cohort, the TT genotype was not significantly over-represented in the ATP group ($P = 0.085$; OR = 1.68; 95% CI 0.95, 2.98). The TT genotype remained significantly over-represented when both cohorts (AUS + SA) were analysed together ($P = 0.004$; OR = 1.82; 95% CI 1.23, 2.74).

**Discussion**

The main finding of this study was that the GDF5 gene was associated with Achilles tendon pathology within an AUS population, independently, and when combined with an additional SA population. More specifically, individuals with a TT genotype of the functional GDF5 rs143383 variant were twice as likely to develop Achilles tendon pathology ($P = 0.004$; OR = 1.82; 95% CI 1.23, 2.74). In addition, we found no association between the functional TGFB1 rs1800469 variant and Achilles tendon pathology in either cohort.

The effect of GDF5 gene transfer as well as the consequence of GDF-5 deficiency have been well established within animal models [10, 11, 36]. However, to our knowledge, no previous work has reported the possible role of this protein in the pathogenesis of human Achilles tendon pathology. Mikic et al. [27] examined the ultrastructural, compositional and mechanical characteristics of mutant GDF-5-deficient mice. Although the Achilles tendons of the mutant GDF-5-deficient mice contained ~40% less collagen, a significantly decreased Achilles tendon tensile strength appeared to be the result of compromised material properties, rather than simply a net reduction in collagen [27]. Moreover, small and irregularly shaped tendons were also observed within the mutant GDF-5-deficient mice [27].

Chhabra et al. [36] also described functional differences between GDF-5-deficient mice and controls. In their study, mutant GDF-5-deficient mice displayed a short-term delay (1–2 weeks) in Achilles tendon healing. A significantly greater amount of fat was also observed within the repaired tissue of the GDF-5-deficient mice. These findings are consistent with the observations of improved clinical outcome within GDF-5-transduced rat tendons [10, 11].

The functional effects of the GDF5 rs143383 variant in modulating GDF5 expression has been well established [31–33]. Differential allelic expression has revealed that the T allele had up to 27% lower expression when
compared with the C allele [32]. The reduced expression of the T allele has been reported across a wide range of soft tissues [33]. The observations from these functional studies, as well as the above-mentioned effects within mutant GDF-5-deficient animals, are in agreement with our current novel finding that the TT genotype of the GDF5 rs143383 variant is associated with an increased risk of Achilles tendon pathology.

The functional effect of the TGFB1 rs1800469 variant has also been determined [17, 20]. Grainger et al. [20] determined the genetic contribution to the control of plasma levels of TGF-β1, and it was also determined that individuals with a TT genotype of this variant produced twice the amount of acid-activated TGF-β1, when compared with CC individuals. Further investigation has attributed the change in TGF-β1 plasma levels to transcriptional suppression due to the transcription complex, AP1, binding to the C allele of the TGFB1 rs1800469 variant [17]. Therefore, the finding in our current study, that the TGFB1 rs1800469 variant was not associated with Achilles tendon pathology is an additional important finding. However, we may not exclude the possibility that additional sequence variants within the TGFB1 gene modify the risk of Achilles tendon pathology.

One strength of the current study was that the selected sequence variants were investigated within two separate population groups. Although the GDF5 rs143383 variant was only associated with Achilles tendon pathology within the AUS population, the allele frequencies among the SA ATP and CON groups were similar to that of the AUS groups. The lack of significance within the SA population may be attributed to a lack of statistical power. Although the SA cohort contained a higher absolute number (and proportion) of subjects with Achilles tendinopathy, the effect size we detected in the AUS cohort would require a greater sample size in the SA cohort for replication (due to the differing allele frequencies and proportions of diseased subjects). Furthermore, within the SA cohort, similar genotype frequencies were observed between patients diagnosed with chronic Achilles tendinopathy and patients with Achilles tendon ruptures.

Although the current study reports an association between the GDF5 rs143383 variant and Achilles tendon pathology, it is possible that other variants within this gene may further influence the risk of Achilles tendon pathology. This should, however, be the focus of future studies. For example, evidence has been presented that GDF5 gene expression is influenced by two additional polymorphisms, dependent on (a 5′ UTR SNP, rs143384) and independent (a 3′ UTR SNP, named 2250ct) of the GDF5 rs143383 variant [33]. However, neither of these additional polymorphisms was associated with risk of OA within large population case–control studies, which had previously identified the GDF5 rs143383 variant as a risk factor for this pathology [31, 32]. The fact that the ATP and CON groups within the SA and AUS cohorts were not matched for all physical characteristics is a potential limiting factor. The relatively small sample size of the AUS and SA TEN groups may also be a limitation. Furthermore, although both SA and AUS cohorts were in HWE, the possibility of population stratification may not be excluded. Future genetic association studies in this field should, therefore, contemplate genotyping a panel of ancestry-informative markers across the genome to exclude the possibility of population stratification [37].

In conclusion, this study suggests that individuals with a TT genotype of a functional SNP within the 5′ UTR of the GDF5 gene (rs143383) are approximately twice as likely to develop Achilles tendon pathology. Therefore, we propose a role for GDF-5 in the pathogenesis of Achilles tendon pathology. Moreover, we propose that this genetic sequence variant should be included in multifactorial models developed to identify individuals predisposed to Achilles tendon pathology. This specific genetic variant may in addition also benefit future individualized clinical and therapeutic approaches to the treatment and management of Achilles tendon pathology.

**Rheumatology key messages**

- We report that a functional GDF-5 variant is associated with Achilles tendon pathology.
- This finding highlights the role of GDF-5 in the pathogenesis of Achilles tendon pathology.

**Acknowledgements**

The authors would like to thank Dr G.G. Mokone for assistance in the recruitment of the SA ATP cohort.

**Funding:** This work was supported by the Leverhulme Trust (09851/B to M.P.); the National Research Foundation (NRF) of South Africa (FA2005021700015 and FA2007032700010 to M.C.); the University of Northampton, the University of Cape Town and the South African Medical Research Council (MRC).

**Disclosure statement:** The authors have declared no conflicts of interest.

**References**

Michael Posthumus et al.


