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Original research

Additional evidence supports association of common genetic variants in *MMP3* and *TIMP2* with increased risk of chronic Achilles tendinopathy susceptibility

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ABSTRACT

Objectives: To systematically evaluate the effects of matrix metalloproteinase-3 (MMP3) and tissue inhibitor of metalloproteinase-2 (TIMP2) on chronic Achilles tendinopathy (AT) susceptibility. Chronic AT is one of the most prevalent and severe injuries in athletes. Early studies suggested that tendon extracellular matrix (ECM) may be involved in the pathogenesis of chronic AT. MMP3 is an important member of the MMP family and is important to ECM integrity. In addition, tissue inhibitor of metalloproteinase-2 (TIMP2) can indirectly limit the activity of MMP3 activity.
Design: Case-control genetic association study.
Methods: A total of 1084 chronic AT patients and 2188 controls with Chinese Han ancestry were recruited. Twenty-one SNPs, 4 mapped to *MMP3* and 17 mapped to *TIMP2*, were selected and genotyped. Genetic association analyses and eQTL analyses were performed. In addition, we also examined the potential effects of epistasis using a case-only study design.
Results: Two SNPs, rs679620 (OR = 0.82, $P = 0.0006$, *MMP3*) and rs4789932 (OR = 1.2, $P = 0.0002$, *TIMP2*) were identified to be significantly associated with chronic AT risk. No significant results were obtained from epistasis analyses. SNP rs4789932 was identified to be strongly associated with the gene expression level of *TIMP2* in two types of human tissues: atrial appendage ($P = 0.0003$) and tibial artery ($P = 0.0009$).
Conclusions: We have identified genetic polymorphisms in *MMP3* and *TIMP2* to be significantly associated with chronic AT risk. Further eQTL analyses indicated that SNP rs4789932 of *TIMP2* was related to the gene expression levels of *TIMP2*. These results suggest important roles for *MMP3* and *TIMP2* in the pathophysiology of chronic AT.
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Practical implications

- Athletes with G allele at rs679620 (*MMP3*) would have an increased risk of chronic AT.
- Athletes with C allele at rs4789932 (*TIMP2*) would have an increased risk of chronic AT.
- If further research and wider replications are conducted to validate in larger population-based studies, it would be useful to clarify the relationship between *MMP3* and *TIMP2* and chronic AT risk, which would provide intriguing new insight into the multifaceted management, treatment and prevention of chronic AT for sport and exercise practitioners.

1. Introduction

Chronic Achilles tendinopathy (AT) is a common clinical disease among athletes and in the general population, which can cause pain and even disability.[1] Epidemiological studies have reported that the lifetime prevalence of AT has been reported to be as high as 11%.[2] Previous etiologic studies suggested that environmental factors and self-diseases play an important role in the occurrence and development of chronic AT. Repetitive forces, overuse,[3] amyloidosis, and rheumatologic diseases[4] all can increase risk for AT. However, a strong genetic component to chronic AT has been firmly established.[5,6] So far, many susceptibility genes have been identified including collagen V alpha 1 (COL5A1) and tenascin C protein (TNC),[7,8] which may alter the tendon extracellular matrix (ECM) structure and/or regulation. These findings suggest that ECM may be involved in the pathogenesis of chronic AT.

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With the rapid development of high-throughput sequencing, more and more susceptibility variants of complex diseases have been identified.[11–13] Accumulating evidence suggests that chronic AT is caused by the disruption of extracellular matrix (ECM) homeostasis,[14] and one of the main steps is the pathological degradation induced by enzymes, such as matrix metalloproteinases (MMPs).[15] MMP3, an important member of the MMP family, is of particular importance to the ECM integrity by catalytically degrading multiple structural proteins including types II, IV, V, IX, X collagens, fibronectin, and aggrecan.[16] In addition, MMP3 can also activate other MMPs to work on the ECM. Previous studies have indicated that lower MMP3 levels exist in chronic AT patients when compared to control patients at the levels of mRNA and protein.[17,18] Therefore, MMP3 may contribute to the risk for chronic AT by affecting ECM homeostasis. Tissue inhibitor of metalloproteinase (TIMPs) is an inhibitor of MMPs. At present, the dynamic balance between MMPs and TIMPs is an important factor in maintaining the degradation of ECM.[19] Researchers have found that TIMP2 can indirectly limit the activity of MMP3 activity.[20] Decreasing the level of TIMP2 has been demonstrated in the human degenerate Achilles tendon compared to healthy tissue.[21] Hence, both MMPs and TIMPs may affect ECM and eventually lead to AT.

A case-control study reported a strong association of the single nucleotide polymorphism (SNP) rs679620 in *MMP3* with AT in South Africans, with the G allele as the risk allele (OR = 2.5, $P = 0.010$).[22] Another study in the British population also demonstrated that the SNP rs679620 was significantly associated with AT in males.[23] These results further proved that *MMP3* may be involved in the pathogenesis of AT. Two studies report the association between AT and the *TIMP2* gene. Khoury et al. found a significant association between *TIMP2* rs4789932 and AT in Australians ($P = 0.016$) and the British ($P = 0.038$).[23,24] However, these available studies have only shown that MMP3 and TMIP2 are associated with AT in Caucasians. Given that different ethnic populations may exhibit genetic heterogeneity, additional studies using more samples from different populations are needed to confirm these associations. Therefore, in the present study, we aimed to study whether *MMP3* and *TIMP2* are associated with the susceptibility of AP in the Han Chinese population.

2. Methods

A total of 3272 unrelated Han Chinese individuals comprising 1084 chronic AT patients and 2188 controls were recruited from Honghui Hospital of Xi'an Jiaotong University between June 2014 and May 2018. All patients with chronic AT were diagnosed based on gradual, progressive pain in the posterior lower limb, localized to the Achilles tendon by examination, for no less than 6 months. In addition, the inclusion criteria had to meet one of the following criteria: (1) morning pain or stiffness in the Achilles tendon area, (2) history of Achilles tendon swelling, (3) palpation tenderness of the Achilles tendon, (4) nodular thickening of the lesion with Achilles tendon, or (5) movement of the Achilles pain area with plantar-dorsiflexion. The examination of soft tissue ultrasound was performed to confirm the diagnosis of chronic AT. Meanwhile, unrelated healthy controls without any history of any tendon or ligament pathology were recruited from the same hospital. All subjects were physically active and born in the local area. Unrelated Han Chinese individuals without migration history were randomly chosen. Subjects who had been diagnosed with any connective tissue disorders or any other systemic diseases potentially associated with Achilles tendon pathology were excluded from the study. Data on general characteristics and clinical information were obtained from medical records or questionnaires (Supplemental Table S1). Significant difference could be identified for BMI between cases and controls ($P < 0.001$), although the difference was very tiny. Distributions of age, gender, smoking status and alcohol use were balanced between chronic AT patients and controls. Written informed consent was obtained from subjects. This research was performed in accordance with the ethical guidelines of the Declaration of Helsinki (version 2002) and was approved by the Ethics Committee of Honghui Hospital of Xi'an Jiaotong University.

SNPs located within the *MMP3* and *TIMP2* gene regions, with a minor allele frequency (MAF) ≥ 0.05, were searched in the 1000-genomes CHB database. $r^2 \geq 0.8$ and $r^2 \geq 0.5$ were used as the cutoff criteria for *MMP3* and *TIMP2* in pairwise tagging, respectively. In total, 21 tagging SNPs were selected for further genotyping (4 mapped to *MMP3* and 17 mapped to *TIMP2*). The basic information for these 21 tagging SNPs is summarized in Supplemental Table S2. Most of these SNPs were located at noncoding regions. Genomic DNA was extracted from peripheral blood leukocytes according to the manufacturer's protocol (Genomic DNA Kit, Axygen Scientific Inc., CA, USA). The selected tagging SNPs were genotyped using the high-throughput Sequenom MassARRAY platform (Sequenom, San Diego, CA, USA) according to the manufacturer's protocol. The results were processed using Sequenom Typer 4.0 software to generate genotypic data. For quality control, the disease state of the sample was unknown throughout the genotyping process. The final genotyping call rate for each SNP was greater than 99%, and the overall genotyping call rate was 99.9%. Subsequently, we randomly selected 5% of the samples for re-genotyping, and the results were exactly the same as before.

We have performed single marker-based association analyses using Plink.[25] Logistic models were fitted to estimate the effect of specific alleles on the risk of chronic AT. BMI was included as a covariate because of its unbalanced distribution between cases and controls. Linkage Disequilibrium (LD) blocks were constructed based on an algorithm proposed by Gabriel et al.[26] Haplotype-based association analyses were conducted for each LD block. In addition to genetic association analyses, we also analyzed the potential epistasis effects between *MMP3* and *TIMP2*. Case-only design was used and SNP pairs between the two candidate genes were tested exhaustively. In general, Bonferroni corrections were applied to multiple comparisons. Therefore, for single marker-based association analyses, the threshold of P values was $0.05/21 \approx 0.002$.

Two bioinformatics tools, RegulomDB (http://www.regulomedb.org/)[27] and SIFT (http://sift.bii.a-star.edu.sg/),[28] were utilized to predict the functional consequences of the significant SNPs. RegulomeDB is an online platform to annotate SNPs using data extracted from the ENCODE project to estimate the biological effects of SNPs on regulations of gene expression. On the other hand, SIFT could predict whether an amino acid substitution affects protein function based on sequence homology and the physical properties of amino acids. In addition to these bioinformatics tools, we also examined the potential eQTL effects of the significant SNPs in multiple human tissues by extracting data from the GTEx database (https://gtexportal.org/).[29]

3. Results

Hardy–Weinberg Equilibriums (HWE) were tested in controls, and all SNPs were in HWE (Supplemental Table S2). Two SNPs, rs679620 (OR = 0.82, $P = 0.0006$, *MMP3*) and rs4789932 (OR = 1.2, $P = 0.0002$, *TIMP2*), were identified to be significantly associated with the disease status of chronic AT (Table 1). The full results of single marker-based association analyses are summarized in Supplemental Table S3. Two LD blocks in *MMP3* and six LD blocks in *TIMP2* were constructed (Fig. 1), and the r^2 values of the SNP pairs based on 21 selected SNPs were presented in Supplemental Figure S1. As shown, the r^2 values were 0.64 between rs538161727

Table 1
Significant results of single marker-based association analyses.

SNP	Study subjects	Genotypic tests (%)			χ^2	*P*	Allelic tests (%)[a]		Tested allele	*T*	OR 95% CI[a]	*P*[a]
		AA	AG	GG			A	G				
rs679620	Cases	78 (7)	460 (42)	546 (51)			616 (28)	1552 (72)			0.82	
	Controls	234 (11)	950 (43)	1004 (46)	12.5	0.0019	1418 (32)	2958 (68)	A	−3.4	0.74–0.93	0.0006
		CC	CT	TT			C	T				
rs4789932	Cases	166 (15)	488 (45)	430 (40)			820 (38)	1348 (62)			1.20	
	Controls	249 (11)	950 (43)	989 (46)	14.4	0.0008	1448 (33)	2928 (67)	C	3.7	1.11–1.37	0.0002

[a] Logistic models were fitted for allelic tests to adjust BMI of study subjects.

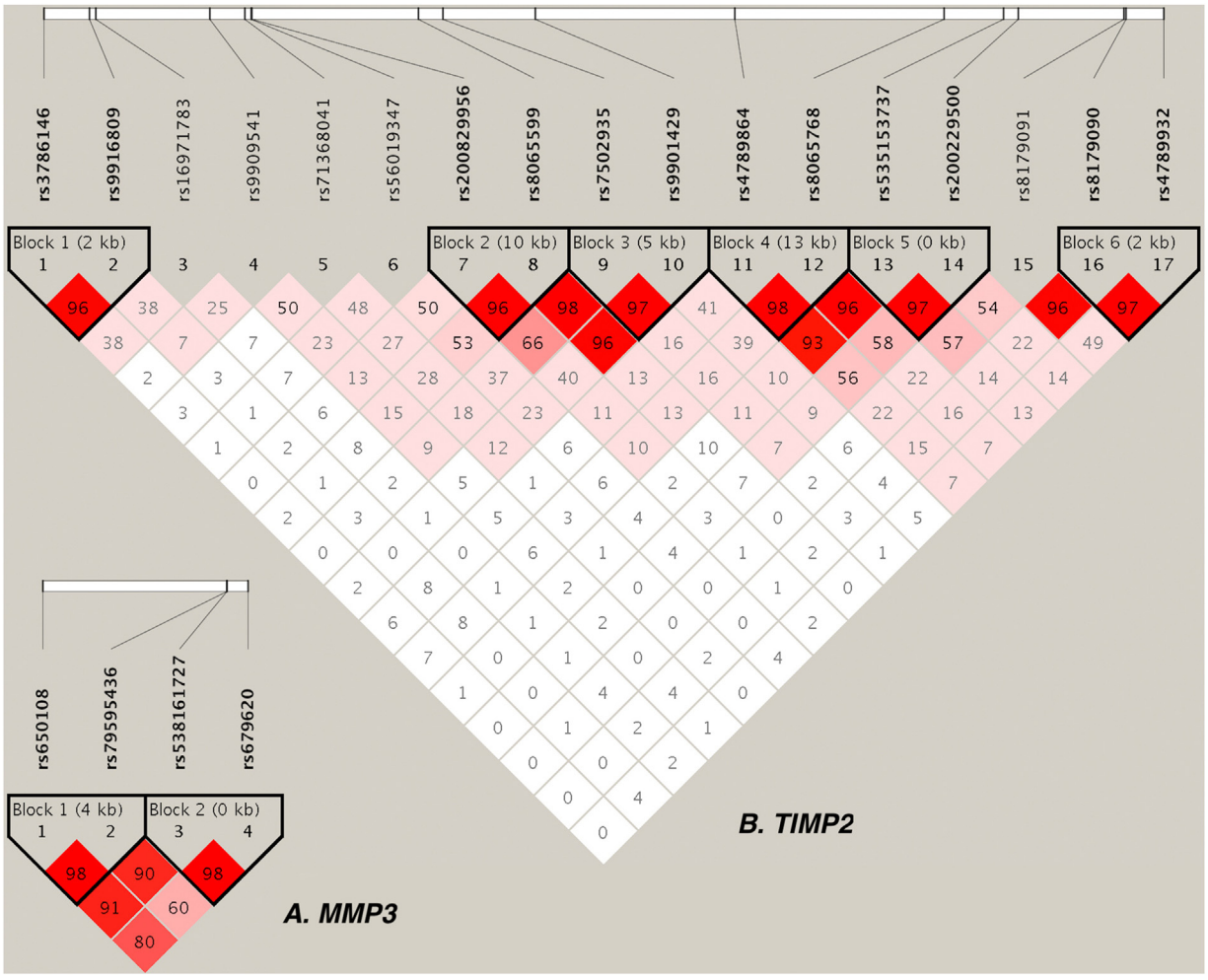

Fig. 1. LD structure based on the 21 selected SNPs. The LD blocks are indicated as shaded pentagons. (A) *MMP3*, left bottom; (B) *TIMP2*, middle. The digits within each diamond shape indicate the *D'* values of the SNP pairs.

Table 2
Significance of haplotype-based association analyses.

LOCUS	HAPLOTYPE	F_A	F_U	χ^2	DF	*P*	SNPs
MMP3	OMNIBUS	–	–	31.42	2	1.50×10^{-7}	rs538161727|rs679620
MMP3	TA	0.28	0.32	11.75	1	0.0006	rs538161727|rs679620
MMP3	TG	0.12	0.08	25.77	1	3.85×10^{-7}	rs538161727|rs679620
MMP3	CG	0.60	0.59	0.03	1	0.8618	rs538161727|rs679620
TIMP2	OMNIBUS	–	–	21.52	2	2.13×10^{-5}	rs8179090|rs4789932
TIMP2	GC	0.18	0.18	0.17	1	0.6768	rs8179090|rs4789932
TIMP2	CC	0.20	0.16	19.61	1	9.47×10^{-6}	rs8179090|rs4789932
TIMP2	CT	0.62	0.67	14.67	1	0.0001	rs8179090|rs4789932

F_A: haplotype frequency in cases; F_U: haplotype frequency in controls; DF: degree of freedom.

and rs679620, and 0.39 between rs8179090and rs4789932. Further haplotype-based association analyses showed that LD blocks (rs538161727-rs679620 in *MMP3* and rs8179090-rs4789932 in *TIMP2*) containing the significant SNPs in single marker-based association analyses were identified to be significantly associated with the disease status of chronic AT (Table 2). Sixty-eight SNP pairs

were tested in our epistasis analyses. No significant results could be obtained from epistasis analyses (Supplemental Table S4).

RegulomeDB has a scoring system, ranging from 1 to 7, to evaluate the functional consequences of SNPs. A lower score represents higher functional significance of the specific SNPs. Both SNPs were tested in RegulomeDB. SNP rs4789932 has a score of 4, while there were no data to show for SNP rs679620. Therefore, the functional significance of SNP rs4789932 was very limited. SNP rs679620 was a nonsynonymous coding SNP and was examined in SIFT. The results showed that SNP rs679620 was a "tolerated" change and therefore might have very limited functional consequence through alteration of the protein sequence.

eQTL data were extracted for SNP rs4789932 and rs679620 from 47 human tissues.[29] SNP rs4789932 was identified to be significantly associated with the gene expression level of *TIMP2* in two types of human tissues (Supplemental Figure S2, Supplemental Table S5): atrial appendage ($P = 0.0003$) and tibial artery ($P = 0.0009$). No significant results were obtained for SNP rs679620 on *MMP3* (Supplemental Table S6).

4. Discussion

In the present study, we identified significant association hits from two candidate genes *MMP3* and *TIMP2* with disease status of chronic AT. Our findings have shown that after being adjusted for BMI, the odds of A allele of rs678620 in AT cases was 18% lower compared with that in controls, while that of the C allele of rs4789932 in AT cases was 20% higher compared with that in controls. Given that it is difficult to draw convincing conclusions only from SNP-based association analysis,[32–34] we performed haplotype-based association analyses. Further haplotype-based association analyses have validated these findings based on single-marker association analyses. Haplotypes containing the two significant hits have also showed significant associations with disease status of chronic AT. To the best of our knowledge, our study was the first genetic association study focusing on chronic AT and *MMP3* and *TIMP2* in Han Chinese population. A previous study based on South Africans has reported *MMP3* gene to be a significant loci,[22] while one study based on Caucasians has suggested a weak association signal for *TIMP2* gene.[35] In addition, another study based on Caucasians has showed that both loci are associated with AT.[23] On the other hand, our study recruited more than 3000 samples and therefore could provide sufficient statistical power to more accurately estimate the potential effects of our selected SNPs. However, it is worth noting that our study was based on the general population, and the results might be different from those of some other studies based on a cohort of athletes.

TIMPs inhibit MMPs and therefore the products of *MMP3* and *TIMP2* might directly interact and work together to affect the risk of chronic AT. However, our epistasis analyses did not show any signs of interactions between the two loci. This could be, at least partly, due to the limited statistical power in our study design in detecting epistasis effects. In this study, we have applied an exhaustive strategy to test all the candidate SNP pairs. Although this "hypothesis-free" strategy enabled us to cover all the genotyped SNPs, it would cause severe problem of multiple comparisons. In addition, early functional studies have provided evidence for the coexpression of *MMP3* and *TIMP1* and *TIMP3* (but not *TIMP2*) in multiple types of cell lines.[36] These findings indicated that the interactions between *MMP3* and *TIMP2* might be weaker compared to *TIMP1* or *TIMP3*. In future studies, it would be good to examine the epistasis effects between *MMP3* and *TIMP1* or *TIMP3* instead of *TIMP2*.

SNP rs678620 in *MMP3* was a nonsynonymous change. However, the results of our bioinformatics analyses showed that this change in amino acid sequence would not significantly alter the biological function of the MMP3 protein. Further eQTL analyses based on GTEx data also showed that the alleles of this SNP could not significantly affect the gene expression levels of *MMP3* in multiple human tissues. Based on these results, the functional significance of SNP rs678620 was very limited. The association signal identified between this SNP and disease status of chronic AT could just be a surrogate of some underlying ungenotyped variants. Therefore, in future studies, a sequencing-based study design might be more informative to help unravel the genetic structure of *MMP3* and its relationships with the disease risk of chronic AT.

On the other hand, SNP rs4789932 was located on the intronic region of *TIMP2*, and thus does not affect the protein structure of *TIMP2*. Nevertheless, the results of eQTL analyses showed that SNP rs4789932 was significantly associated with *TIMP2* gene expression in two types of human tissues: atrial appendage and tibial artery. This finding indicated that SNP rs4789932 might affect the risk of chronic AT by regulating the gene expression level of *TIMP2*. However, we need to be careful in interpreting these eQTL results based on the GTEx database. A potential limitation of these eQTL analyses is that all of these gene expression data were collected from human tissues of healthy subjects. No additional expression data from chronic AT patients could be added, and thus the current data from healthy subjects does not represent the status in chronic AT patients. Additional functional studies are needed in the future to explore the functional consequences of SNP rs4789932 on *TIMP2*.

Our study suffered from several limitations. First, population stratification might confound the results and cause false positive signals. As a candidate gene-based association study, we cannot apply some standard procedures such as principal component analysis to adjust this confounder. Nevertheless, we applied strict criteria in the recruitment process to ensure a higher degree of homogeneity in the genetic background of our study subjects. In addition, only a couple common variants were selected for genotyping in this study, and underlying variants with true effect may have been missed.

5. Conclusion

In this study, we have identified genetic polymorphisms in *MMP3* and *TIMP2* to be significantly associated with chronic AT risk. Further eQTL analyses indicated that SNP rs4789932 of *TIMP2* was related to the gene expression levels of *TIMP2*. These results suggest important roles for *MMP3* and *TIMP2* in the pathophysiology of chronic AT. Therefore, further research and wider replications should be conducted to validate in larger, preferably population-based, studies to elucidate the exact molecular basis of the relationship between *MMP3* and *TIMP2* and chronic AT risk, which would help to reveal the etiology of chronic AT and provide intriguing new insight into its pathogenesis.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jsams.2019.05.021.

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