Novel Tenascin-C Haplotype Modifies the Risk for a Failure to Heal After Rotator Cuff Repair

Rainer Kluger,*† MD, Klaus R. Huber,†‡ PhD, Philipp G. Seely,† MD, Christian E. Berger,† MD, and Florian Frommlet,§ PhD
Investigation performed at the Orthopedic Department, SMZOst Donauspital, Vienna, Austria

Background: Several single-nucleotide polymorphisms (SNPs) in the *TNC* gene have recently been found to be associated with degenerative rotator cuff tears.

Hypothesis: Exonic SNPs in the *TNC* gene are related to the risk for a failure to heal after rotator cuff repair.

Study Design: Case-control study; Level of evidence, 3.

Methods: A total of 302 patients from the Vienna area and European Caucasian ancestry underwent mini-open rotator cuff repair for a full-thickness superior or posterosuperior tear and were assessed for the integrity of the repair 1 year postoperatively with a real-time 7.5- to 10-MHz ultrasound linear array transducer. Outcomes were classified as intact (complete footprint coverage), small (<200 mm^2), or large (≥ 200 mm^2) recurrent defect. Patients were genotyped for 15 previously identified risk SNPs within a 49-kbp segment of the *TNC* gene with the KASP genotyping technology or the Ion-Torrent Personal Genome Machine System.

Results: All recurrent defects were atraumatic failures, and the overall failure rate was 39.7%. Of the traditional risk factors, only the initial tear size was significantly associated with a failure to heal. In a multinomial logistic regression model, the T allele at rs1138545 [C>T] was protective for a large recurrent defect (odds ratio = 0.16; 95% CI, 0.09-0.31). The role of rs1138545 was further backed by haplotype analysis, which showed that the combination of the C allele at rs1138545 [C>T], the A allele at rs2104772 [A>T], and the G allele at rs10759752 [A>G] formed the risk-related haplotype [CAG]. The CAG haplotype was associated with large recurrent defects ($P < .0001$; haplotype frequency, 0.394; haplotype score, 4.518). Exonic marker rs1138545 transcribed into all isoforms of the TNC protein, whereas exonic marker rs2104772, which has been associated with Achilles tendinopathy before, transcribed only into large isoforms of the TNC protein.

Conclusion: Recurrent defects after rotator cuff repairs are clinically relevant, and a heritable component of the disorder is plausible on the basis of a genetic association with 8 TNC variants. Characterization of TNC sequences that favor tendon healing will help engineer new products in regenerative medicine.

Keywords: *TNC* gene variants; rotator cuff repair healing; SNP; recurrent rotator cuff defects

The prevalence of degenerative rotator cuff tears (RCTs) increases with advancing age.[35] When RCTs are symptomatic and nonoperative management fails, a tendon-to-bone repair is performed surgically. However, despite advances in surgical techniques in the past decades, the overall healing rate after rotator cuff repairs has not improved.[17,21] Further investigations of potential predictors of a failure to heal are justified because the loss of structural integrity at the repair site has been associated with poor clinical outcomes—specifically, among younger, more active patients and patients who have labor-intensive occupations and when outcome measures include strength or active shoulder motion.[12,13,17,20,21,24] Traditional predictors of a failure to heal are fatty infiltration and muscle atrophy (the chronicity of the tear) as well as the initial tear size.[11,17,21,28] It is obvious that the required high initial fixation strength,[10] as well as a close proximity of tendon and bone during at least 12 weeks after the repair (when gap formation is most likely),[8,10,13,20] is more difficult to maintain postoperatively in large and retracted tears than in smaller, less retracted tears. Older age is another traditional predictor of a failure to heal.[11,17,21,28] It is not yet clear whether "age" might comprise a number of risk factors, such as

*Address correspondence to Rainer Kluger, MD, Department of Orthopedics, SMZOst Donauspital, Langobardenstrasse 122, A-1220 Vienna, Austria (email: rainer.kluger@wienkav.at).
†Department of Orthopedics, SMZOst Donauspital, Vienna, Austria.
‡Institute of Laboratory Medicine, SMZOst Donauspital, Vienna, Austria.
§Center for Medical Statistics, Informatics, and Intelligent Systems, Medical University of Vienna, Vienna, Austria.

One or more of the authors has declared the following potential conflict of interest or source of funding: Grant sponsorship for laboratory costs was received from the Department of Orthopedics, SMZOst Donauspital.

The American Journal of Sports Medicine, Vol. XX, No. X
DOI: 10.1177/0363546517729810

systemic osteoporosis, which plays an independent role in a failure to heal.[5] Some authors have suggested that the likelihood of a recurrent defect could be solely calculated from initial tear size and the patient's age.[15,26] However, in addition to these traditional risk factors, local "tendinosis/tendinopathy" has been identified as an independent predictor for a failure to heal.[3-5,11,15,24,27] Indeed, healing is significantly impaired in tendons showing increased signal intensity on magnetic resonance imaging,[5] an inferior histologic appearance,[4] or intraoperative signs of degeneration, such as swelling, fraying, and friability.[11,15,24] Moreover, degeneration is mirrored by the molecular pathologic level, where certain gene expression profiles are indicative of an increased risk to fail.[3,27]

We recently took an important next step by identifying a risk haplotype in the *TNC* gene associated with degenerative RCTs.[14] TNC protein is a cell adhesion–modulating ECM glycoprotein, only sparsely expressed in the healthy adult organism. TNC is present in connective tissues that bear tensile strength and in certain stem cell niches, including the bone marrow.[7] Mechanical or chemical injury, including mechanical overload of muscle and tendons, leads to rapid upregulation of TNC protein synthesis in affected tissues.[33] TNC protein is composed of a series of epidermal growth factor–like repeats, fibronectin type III (FNIII)–like repeats, and a C-terminal fibrinogen–like globular domain. Of note, 8 of the FNIII repeats (FNIII 1-8) are always present, but 9 potentially expressed FNIII repeats (FNIII A1, A2, A3, A4, B, AD2, AD1, C, D) are found only in large isoforms of TNC protein. In larger isoforms, TNC aids cell migration and dynamic tissue organization of the ECM, while the smaller, proadhesive isoforms mediate stability of newly formed tissues through binding to, for example, fibronectin.

We studied the long-term survival probability of rotator cuff repairs[13] and showed that the size of recurrent defects is critical for the clinical outcome when assessed through shoulder scores. More recently, we conducted a genetic association study and identified 15 single-nucleotide polymorphisms (SNPs) in the *TNC* gene that modulate the risk for degenerative RCTs.

In the current study, we reassessed these 15 TNC markers and genotyped 92 patients from our previous long-term survival study[13] as well as an additional 210 patients with repaired degenerative RCTs. All patients received the same surgical method. In this independent cohort of 302 patients, several SNPs that have been related to degenerative RCTs were significantly associated with a failure to heal after rotator cuff repair.

Abbreviations and terms used in this article are defined in Table 1.

TABLE 1
Abbreviations and Terms Used

Abbreviation/Term	Definition
ECM	Extracellular matrix
FNIII 5	Fibronectin type III–like repeat number 5
iTS	Initial tear size
kbp	Kilobase pairs (a measure for the length of a DNA segment)
LD	Linkage disequilibrium; nonrandom distribution of alleles as a result of selection pressure
LG	Linkage group expressed as the squared correlation coefficient (r^2)
MAF	Minor allele frequency
Major allele	Sequence or gene that is higher in frequency in a reference population
Minor allele	Sequence or gene that is less frequent in a reference population
r^2	Squared correlation coefficient between pairs of biallelic (SNP) loci
Risk haplotype	Genetic background associated with higher risk
SNP	Single-nucleotide polymorphism
Exonic SNP	Genetic variant located in an exon
Intronic SNP	Genetic variant located in an intron

METHODS

Study Population and Genetic Background

After approval from the ethics committee of the Medical University Vienna (reference EK: 06/017-VK), a prospectively collected database was retrospectively reviewed, and 437 consecutive cases of full-thickness superior or posterosuperior RCTs (supraspinatus or combined supraspinatus and infraspinatus) that had undergone arthroscopically assisted mini-open repair by the first author (R.K.) at a single institution between January 1999 and October 2005 were identified. Patients who had an all-arthroscopic repair or a repair for a partial tear during the same period were not considered eligible. Of the 437 consecutive cases, patients who had an irreparable full-thickness tear, an additional subscapularis tear, symptomatic arthritis of the acromioclavicular joint, or any previous surgery in the same shoulder were excluded. Patients were also excluded if the cuff tear was combined with a history of calcifying tendinitis, trauma, or systemic disease/inflammatory condition. By this method, 308 patients met the inclusion criterion. Before participation in this study, 302 participants gave informed written consent for the postoperative collection of a blood sample and its storage and later genetic testing. Note that the study population comprises 92 patients from our long-term rotator cuff repair survival study.[13] There were 54 tears (18%) with a size <200 mm^2 (isolated supraspinatus; 22 left shoulders, 32 right shoulders) and 248 tears (82%) with a size of 200 to 1400 mm^2 (combined supraspinatus and infraspinatus; 117 left shoulders, 131 right shoulders). The 302 patients from the Vienna area and European Caucasian ancestry (mean age, 59.4 $\pm$ 8.9 years; 48% female) were genotyped for 15 SNPs in the *TNC* gene that had been identified as risk factors for degenerative RCTs in a previous study.[14] The mean body mass index was 25.4 kg/cm^2 (range, 18.1-43.0 kg/cm^2); 159 patients (52.6%) had involvement of the dominant extremity; and 18% of the patients were smokers at the time of surgery.

Clinical Variables

The included demographic variables were age, sex, arm dominance, body mass index, symptom duration, preoperative Constant and American Shoulder and Elbow Surgeons score, and ultrasound or magnetic resonance imaging examination. All participants who enrolled in this study completed a medical history questionnaire, including medication use, personal history of systemic disease and inflammatory disease, steroid injection and smoking status, history of tendon injury (tendinopathies, tendon ruptures), and sports participation.

Description of Treatment or Surgery

All patients had pain for >5 months despite an appropriate trial of nonoperative therapy. The mean duration of symptoms was 7 months. Surgery was performed between 30 and 90 days after first contact with the patient. The surgical technique, intraoperative tear size measurement, calculation of the tear size in square millimeters, and postoperative management have been described.[13] For further information on repair technique, see the Appendix (available in the online version of this article). All clinical follow-up examinations were done by resident orthopaedists according to a protocol described elsewhere,[13] but these results are not included in this genetic study. Each clinical follow-up also included an ultrasound examination. Sonograms were performed and evaluated by an experienced orthopaedist. The 1-year sonographic follow-up examination was used to assess the integrity of the rotator cuff repair and the size of the recurrent defect. No patient missed the 1-year follow-up. Structural outcome was classified as either intact or a recurrent defect <200 mm^2, thus representing only the supraspinatus footprint or a recurrent defect ≥ 200 mm^2.

Structural Outcome Measurement

Soft Tissue Ultrasound. Ultrasonograms were obtained with a real-time 7.5- to 10-MHz linear array transducer on a Sonoline G20 machine (Siemens). Details of the standard protocol for the examination technique have been described.[13]

Assessment of Sonograms. Ultrasonography thermal paper images of each patient were reviewed retrospectively and independently by 2 radiologists who were blinded to the patient's clinical findings at follow-up. All images were then re-reviewed jointly to achieve consensus. When a consensus could not be reached between reviewers, the real-time impression found by the sonographers was used as the final arbiter. A questionnaire was completed noting the presence of an intact cuff or a full-thickness tear according to established criteria.

Criteria for Ultrasound Diagnosis of RCTs. Rotator cuffs were scored as intact, or a diagnosis of full-thickness tear (rerupture) was made. Criteria for a rerupture were (1) a hypoechoic zone that extended through the entire substance of the cuff, (2) a segmental or complete loss of rotator cuff substance with visible margins of a tear, (3) no visualization of the cuff tissue, or (4) if a focal depression was present into which the deltoid muscle could be compressed manually to separate the torn ends.[13] A focal heterogeneous hypoechogenicity (distinct mixed hyperechoic and hypoechoic defect) in the rotator cuff substance, which is a sign of a partial-thickness tear, was classified as a rerupture.

Measurement of Recurrent Defects. The anteroposterior width (base of tear) and the mediolateral depth (retraction of tear) of the recurrent defects were measured sonographically, and for simplification, a U-shaped tear was assumed. The recurrent defect area was then considered as rectangular (area = base $\times$ height). For statistical calculations, recurrent defects were also classified as either <200 mm^2 or ≥ 200 mm^2. We think that this categorization is reasonable because, according to a recent study,[26] once the anteroposterior width of a recurrent defect reaches 20 mm (hence resulting in a maximum area of 200 mm^2 given our calculation method and assuming a mean retraction of 10 mm), infraspinatus fibers are involved to an extent that is clinically relevant.[26]

SNP Selection. This study reports the genotypes and allele frequencies at 15 SNPs (see Table 2) in the *TNC* gene that had recently been found to be associated with degenerative RCTs.[14] The NCBI database (http://www.ncbi.nlm.nih.gov/) was used for annotation and positioning of the SNPs.

DNA Extraction. Genomic DNA was extracted from white blood cells with the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich) according to the manufacturer's instructions.

Genotyping. For 220 patients, genotyping was performed with KASP genotyping technology at the LGC Genomics Laboratories (for details, see http://www.lgcgroup.com/our-science/genomics-solutions).

For 85 patients, genotyping was performed in the SMZOst Institute of Laboratory Medicine. Polymerase chain reaction was performed on an ABI Prism 7000 detection system (Life Technologies) with 5$\times$ HOT FIREPol Probe qPCR Mix Plus (ROX, Solis BioDyne; polymerase chain reaction efficiency = 2). Duplicate experiments utilized 2 μL of the sample, 4 μL of master mix, 1 μL of primer mix (Applied Biosystems, Thermo Fisher; for SNPs, see SNP Selection section), and 20 μL of H_2O; the Ct values of the 2 polymerase chain reactions were <0.3 cycles apart. FAM or VIC fluorescence was used as readout. The amplification blots were visually checked, and allelic discrimination was used for the genotyping.

Statistical Analysis

In our analysis, only those SNPs were included for which all 3 genotypes were present in at least 1 of the 3 groups and the frequency of the minor allele in the sample was at least 5%. As a measure of linkage disequilibrium, the squared correlation coefficient (r^2) between pairs of biallelic loci (SNPs) was used. Clusters of linkage groups are reported according to r^2 cutoff values of either 0.85 or 0.58. Potential deviation from Hardy-Weinberg equilibrium was assessed via the usual chi-square test with the R genetics package.[34] The genotyping success rate was at 99%. Owing to the strong linkage disequilibrium patterns, it

TABLE 2
Candidate SNP Information[a]

dbSNP[b]	*TNC* Gene	MapPos	Major > Minor	Global MAF[b]
rs2210108	Intron 9	115073909	[G>A]	0.255
rs1138545	Exon 10/missense	115073620	[C>T]	0.118
rs3789870	Intron 10	115072997	[G>A]	0.255
rs2104772	Exon 17/missense	115046506	[A>T]	0.481
rs11793430	Intron 17	115045841	[G>C]	0.200
rs953288	Intron 17	115043440	[A>C]	0.414
rs10759753	Intron 17	115043015	[A>G]	0.256
rs72758637	Intron 17	115042922	[C>G]	0.120
rs7021589	Intron 17	115042388	[T>C]	0.059
rs1411456	Intron 18	115041603	[G>A]	0.256
rs10759752	Intron 18	115041441	[A>G]	0.450
rs16932078	Intron 22	115034707	[C>T]	0.328
rs72758634	Intron 23	115031134	[G>T]	0.079
rs13321	Exon 24/missense	115030304	[C>G]	0.332
rs7035322	Intron 26	115024894	[C>A]	0.367

[a]The first column corresponds to the SNP code; the second, to the gene annotation; the third, to the position according to GRCh38.p2; the fourth, to the 2 possible variants that occur at this SNP position—major allele (ie, higher in frequency) and minor allele (ie, less frequent); the fifth column, to the global MAFs.
[b]National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) was used for annotation and positioning of the SNPs.

was possible for the majority of missing genotype values to perform imputation with hardly any ambiguity. Consequently, we performed imputation based on genotype information from linkage group members for some analyses, including multiple markers. Because of the small number of missing values, the results are hardly affected by imputation, whereas working with the full data set simplifies multivariate analysis. As a consequence of the strong linkage disequilibrium patterns, it also follows that the Bonferroni rule is too strict to correct for multiple testing. At the strict r^2 cutoff of 0.85, the 15 SNPs can be grouped in 7 clusters, which motivates a quasi-Bonferroni corrected significance level of $\alpha = 0.05/7 \approx 0.0072$. This will still be conservative given the strong correlation observed among all SNPs.

A multinomial logistic regression model (SAS PROC logistic with link function link = glogit) was used to describe the association of each of the 15 SNPs with the structural outcome.

We first determined which covariates were important to include; we then tested each SNP individually at the corrected significance level (0.0072). Here we considered additive SNP effects only, which means that we modeled the effects of the genotype on the outcome (log odds of a recurrent defect) as a linear trend in terms of the frequency of minor alleles.

Finally, a haplotype analysis for the structural outcome was performed with the R haplo.stats package,[31] wherein we applied score tests for the multinomial outcome.

RESULTS

Clinical and Sonographic Results

Of the 302 chronic degenerative RCTs that underwent arthroscopically assisted mini-open repair by a single surgeon (R.K.) at 1 institution, 120 (39.7%) showed a recurrent defect at the time of the 1-year sonographic follow-up examination. In all cases with a recurrent defect, sonography showed the footprint area to be devoid of soft tissue. We did not find recurrent ruptures at the musculotendinous junction. In some sonograms, loose suture material could be visualized near the recurrent defect area. We concluded that in cases with recurrent defects, healing had failed at the tendon-bone interface. The recurrent defect area was <200 mm^2 and thus assumed to be limited to the supraspinatus footprint[26] in 21 shoulders (mean, 110 mm^2; range, 50-180 mm^2) and ≥ 200 mm^2 in 99 shoulders (mean, 747 mm^2; range, 200-1300 mm^2). Of the potentially confounding variables for RCTs, neither arm dominance nor smoking status was associated with initial tear size or with the occurrence or size of the recurrent defect.

SNP Genotype Information

The overall success rate for genotyping was 99.0%. Table 3 provides basic information about the genotype distribution of the 15 assessed candidate SNPs. The assessed *TNC* gene segment (length, 49 kbp) contains groups of SNPs characterized by a complete or nearly complete linkage disequilibrium and similar allele frequencies (Appendix Table A1). Using an r^2 cutoff of 0.85, one obtains 7 clusters (linkage groups [LGs]), 4 of which contain >1 SNP (LG1, LG2, LG5, LG6). Using a threshold of 0.58, one obtains 2 main blocks consisting of LG1, LG5, LG6, and LG7 (LG1#) and LG2 and LG4 (LG2#). In general, SNPs belonging to different main blocks are negatively correlated. rs2104772 (comprising LG3; LG3#) does not strictly belong to any of the 2 main blocks, although it is the only SNP that has strong correlations with SNPs from both blocks. It is positively correlated with LG2# and negatively correlated with LG1#.

TABLE 3
Genotype Frequencies of the Analyzed SNPs[a]

	r^2 Cutoff							
TNC-SNP Code	0.85	0.58	No.	m	Hom1	Hetero	Hom2	MAF
rs2210108	LG1	LG1#	298	4	155	115	28	0.287
rs1138545	LG2	LG2#	302	0	186	105	11	0.210
rs3789870	LG1	LG1#	296	6	148	111	37	0.312
rs2104772	LG3	LG3#	302	0	95	148	59	0.440
rs11793430	LG4	LG2#	300	2	160	114	26	0.277
rs953288	LG5	LG1#	296	6	102	136	58	0.426
rs10759753	LG1	LG1#	299	3	154	117	28	0.289
rs72758637	LG2	LG2#	297	5	181	106	10	0.212
rs7021589	LG2	LG2#	302	0	190	102	10	0.202
rs1411456	LG1	LG1#	299	3	157	114	28	0.284
rs10759752	LG5	LG1#	300	2	98	138	64	0.443
rs16932078	LG6	LG1#	298	4	138	131	29	0.317
rs72758634	LG2	LG2#	298	4	187	101	10	0.203
rs13321	LG6	LG1#	302	0	142	131	29	0.313
rs7035322	LG7	LG1#	296	6	159	118	19	0.263

[a]The first column corresponds to the SNP code position according to GRCh38.p2; the second and third columns, to the linkage group obtained by LD clustering based on cutoff values of 0.85 (LG) and 0.58 (LG#); the fourth, to the number of genotyped SNPs; and the fifth, to missing values. hom1—genotype is homozygous for the major allele; hetero—genotype is heterozygous; hom2—genotype is homozygous for the minor allele. The last column refers to the MAFs within our sample.

Traditional Covariates as Predictors for a Failure to Heal

According to analysis of variance, the covariates body mass index and sex were not associated with the occurrence of a recurrent defect. In contrast, age and initial tear size were both significantly associated with the structural integrity of the repair site at 1 year postoperatively (Appendix Figure A1). We observed that patients with a small recurrent defect also tended to have an initially smaller tear (mean ± SD, 253.8 ± 103.2 mm^2) and were younger (age at the time of surgery, 55.8 ± 7.8 years), whereas patients with a large recurrent defect tended to have an initially larger tear (793.9 ± 335.8 mm^2) and were older (age at the time of surgery, 62.1 ± 7.9 years). Patients without rerupture displayed the full range of the initial tear size (549.9 ± 382.1 mm^2). We previously saw that age and initial tear size are correlated; thus, the effect of age on failure to heal might be mediated through initial tear size. In fact, in a multinomial logistic regression model including age and initial tear size, the effect of age is no longer significant. Consequently, we kept only initial tear size as a covariate in our final analysis.

Genetic Predictors for a Failure to Heal

Table 4 summarizes the results of multinomial logistic regression models where the outcome is explained by initial tear size plus 1 additive SNP effect. The 4 SNPs of LG2 (rs1138545, rs72758637, rs7021589, rs72758634) are strongly associated with failure of healing ($P < .0001$). Furthermore, the 4 SNPs from LG3, LG4, and LG5 are significant at the quasi-Bonferroni-corrected level. All other SNPs apart from rs3789870 show some association with failure of healing but are not significant after correction for multiple testing.

Figure 1 presents detailed results for SNP rs1138545, a representative of LG2 that is of particular interest. The other 3 SNPs of that cluster are all tightly linked with rs1138545 ($r^2 > 0.94$) and have very similar behavior. As expected, initial tear size was a strong predictor for the size of the rerupture, where patients with a large initial tear size tended to also have a large rerupture (odds ratio [OR] = 1.19; 95% CI, 1.10-1.28), whereas patients with a smaller initial tear size tended to also have a smaller rerupture (OR = 0.64; 95% CI, 0.49-0.85). In addition, patients who were homozygous for the C allele at rs1138545 had more frequent failures to heal as well as larger recurrent defects, whereas patients who carried the T allele (as in genotype CT or TT) more often had healed rotator cuff repairs. This protective effect of the T allele (minor allele) is more pronounced for larger reruptures (OR = 0.16; 95% CI, 0.09-0.31) than for small reruptures (OR = 0.40; 95% CI, 0.16-1.01).

Haplotype Analysis

We performed a haplotype analysis including rs1138545, rs2104772, and rs10759752, which serve as representatives for the 3 main linkage disequilibrium blocks (LG1#, LG2#, LG3#) (Table 3 and Appendix Table A1). They also represent the respective subblocks LG5, LG2, and LG3, which, according to Table 4, were associated most strongly with the structural outcome. Table 5 gives the detailed genotype information for these 3 SNPs, and Tables 6 and 7 provide the results of haplotype analysis with the haplo.stats package.[31]

Two highly significant protective haplotypes are characterized by the T allele at SNP rs1138545 [C>T] (Table 6:

TABLE 4
Multinomial Logistic Regression[a]

TNC SNP	r^2 Cutoff, 0.85	*P* Value	OR1	OR2
rs2210108		.0328	1.08736	1.68380
rs1138545	LG2	**<.0001**	0.39907	0.16282
rs3789870		.1813	0.88849	1.39388
rs2104772	LG3	**.0003**	0.72845	0.44060
rs11793430	LG4	**.0002**	1.07700	0.37605
rs953288	LG5	**.0013**	1.42502	1.97628
rs10759753		.0482	1.19297	1.63464
rs72758637	LG2	**<.0001**	0.37285	0.17618
rs7021589	LG2	**<.0001**	0.42964	0.17939
rs1411456		.0319	1.08626	1.68791
rs10759752	LG5	**.0008**	1.48010	2.01525
rs16932078		.0436	0.86869	1.62350
rs72758634	LG2	**<.0001**	0.38131	0.15186
rs13321		.0436	0.86869	1.62350
rs7035322		.0139	1.41278	1.86649

[a]Results from the multinomial logistic regression. The 8 *TNC* variants associated with the postoperative structural outcome of the rotator cuff repair are highlighted. The second column gives the linkage group obtained by LD clustering based on cutoff values of 0.85 for SNPs, which are significant after quasi-Bonferroni correction. *P* values correspond to type 3 analysis of the SNP effect (ie, correcting for initial tear size). Bold indicates significant values. OR1 and OR2 give the odds ratio estimates comparing small rerupture vs healed (OR1) and large rerupture vs healed (OR2). Models including initial tear size and 1 SNP were not further improved by adding additional SNPs.

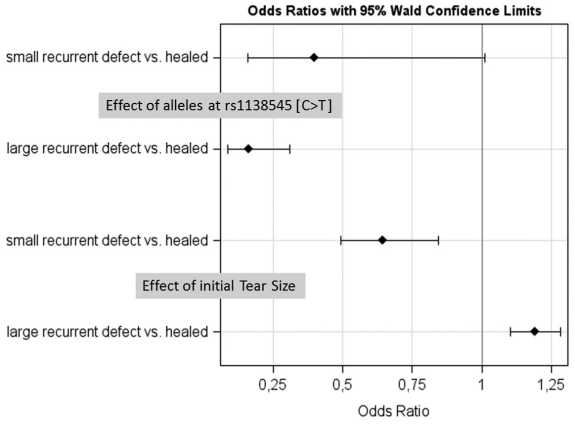

Figure 1. In this multinomial logistic regression model of healing status, we look at small recurrent defect vs healed tendon repair and larger recurrent defect vs healed tendon repair, with SNP genotype as a fixed factor and with initial tear size as a covariate, including odds ratios with 95% confidence intervals. The upper half of the figure illustrates the coefficients of the effect from our main genetic risk locus, SNP rs1138545, for which we see an additive effect of genotypes. (*Additive effect* means that the log odds to have a large rerupture diminishes in a linear way with respect to the frequency of the T allele in the genotype CC, CT, or TT.) This protective effect of the T allele (minor allele) is more pronounced for larger reruptures than for small reruptures. The lower half of the figure illustrates the effect of initial tear size on healing statuses.

TABLE 5
Genotype and Minor Allele Frequency Information[a]

		Recurrent Defect	
TNC	Healed	Small	Large
rs1138545			
No.	182	21	99
CC	0.46	0.71	0.88
CT	0.49	0.24	0.10
TT	0.04	0.05	0.02
Minor T	0.29	0.17	0.07
HWE	0.01	1.00	0.08
rs2104772			
No.	182	21	99
AA	0.25	0.19	0.49
AT	0.52	0.67	0.40
TT	0.23	0.14	0.10
Minor T	0.49	0.48	0.30
HWE	0.65	0.20	0.81
rs10759752			
No.	182	21	97
AA	0.39	0.29	0.36
AG	0.47	0.52	0.42
GG	0.14	0.19	0.22
Minor G	0.37	0.45	0.43
HWE	1.00	0.65	0.21

[a]Genotype and minor allele frequency distributions of 1 representative *TNC* SNP of each main linkage group (LG#1, LG#2, LG#3) that was (after quasi-Bonferroni correction) significantly different between outcome categories. HWE, Hardy-Weinberg equilibrium.

haplotypes 1 and 2). However, the haplotype [CAG] (Table 6: haplotype 6) including the C allele at rs1138545 [C>T], the A allele at rs2104772 [A>T], and the G allele at rs10759752 [A>G] significantly increases the risk for recurrent defects. To assess the contribution of rs2104772 and rs10759752 when controlling for rs1138545, we performed an additional haplotype analysis with initial tear size and rs1138545 as covariates (Table 7). In the resulting score tests, the haplotype [AG] was still significant at the uncorrected level alpha (.05). We concluded that most of the genetic effect is already explained by the genotype of rs1138545 but that the combination of rs2104772 and rs10759752 gives some additional contribution to the overall risk for recurrent defects.

DISCUSSION

Multinomial logistic regression showed that SNP rs1138545 [C>T], located in exon 10 of the *TNC* gene (as well as tightly linked intronic SNPs rs72758637, rs7021589, and rs7275 8634), was the most influential genetic variant associated with a failure to heal at the tendon-bone interface ($P <$ .0001) (Table 4). In particular, it turned out that the presence of the T allele at rs1138545 [C>T], as in patients carrying the genotype CT or the genotype TT (Table 5), was protective against large recurrent defects of rotator cuff repairs (OR = 0.16; 95% CI, 0.09-0.31) (Figure 1). This was confirmed by

TABLE 6
Assessment of Risk Haplotypes and Protective Haplotypes[a]

	TNC SNP					
Haplotype	rs1138545[C>T]	rs2104772[A>T]	rs10759752[A>G]	Hap-Freq	Hap-Score	*P* Value
1	T	T	A	0.178	–5.148	**<.001**
2	T	A	A	0.019	–2.596	**.009**
3	C	T	A	0.218	–0.076	.939
4	C	T	G	0.035	0.227	.821
5	C	A	A	0.142	0.722	.470
6	C	A	G	0.394	4.518	**<.001**

[a]Haplotypes were inferred for the 3 SNPs (rs1138545, rs2104772, rs10759752) and their frequencies estimated (Hap-Freq). Haplotypes are ordered according to the score test statistics (Hap-Score) of a multinomial model including initial tear size as covariate. The last column gives the *P* values of the corresponding score tests. Bold indicates significant values.

TABLE 7
Assessment of Risk Haplotypes[a]

	TNC SNP				
Haplotype	rs2104772[A>T]	rs10759752[A>G]	Hap-Freq	Hap-Score	*P* Value
1	T	A	0.397	–1.239	.215
2	A	A	0.161	–0.801	.423
3	T	G	0.043	–0.573	.567
4	A	G	0.399	1.971	.048

[a]Haplotypes were inferred for the SNPs rs2104772 and rs10759752 and their frequencies estimated (column Hap-Freq). Haplotypes are ordered according to the score test statistics (Hap-Score) of a multinomial model including initial tear size and the genotype of SNP rs1138545 as covariates. The last column gives the *P* values of the corresponding score tests.

the haplotype analysis ($P < .001$), where the main protective haplotype was present in almost 18% of the samples (Table 6: haplotype 1). Conversely, the C allele at SNP rs1138545 [C>T] was identified as the risk allele for this locus. Our data suggest that a failure to heal depends not solely on a combination of traditional risk factors, such as initial tear size, age, and tendon quality.[15,26] Rather than being a process that is purely intrinsic or extrinsic to the tendon itself, the origin of a failure to heal is multifactorial and likely has a polygenic basis.

Until recently, evidence for a heritable predisposition for healing deficiencies of surgically repaired rotator cuffs was limited to 1 report.[32] The authors found that SNP rs17583842 located on chromosome 14 was associated with the phenotype. However, rs17583842 is not located in an exon and hence does not translate into protein. In contrast, our data show for the first time that 2 genetic variants (rs1138545 in exon 10 and rs2104772 in exon 17), which actually translate into TNC protein, are associated with failures to heal after surgical repair. We recently described the remarkable pairwise association of exonic rs1138545 and tightly linked intronic rs7021589 with the degenerative RCT phenotype.[14] The currently found association of these 2 SNPs with a failure to heal in our independent cohort of 302 patients of Caucasian ancestry supports the role of the *TNC* gene in tendon remodeling and repair.[2] The potential functionality of rs1138545 relates to the fact that it translates into the FNIII 5 domain of TNC protein (Figure 2).

TNC protein occurs in a number of isoforms, and the FNIII 5 domain is found in all of them. The particular function of the FNIII 5 domain within TNC protein is to bind heparin, glypican, contactin, neurocan, perlican, lecticans, and periostin, but it also has a high affinity toward a large number of growth factors (eg, PDGF, VEGF, FGF, TGF-β), factors from the neurotropin family, IGF binding proteins, and others with nanomolar binding affinities.[6,7,18,33] The sequestration of soluble factors from the FNIII 5 domain might prolong their half-life, increase their local concentrations, and/or affect their conformation, all of which affect their ability to signal to the cell.[6,18] In addition, intronic rs7021589 (which is in the same linkage group as rs1138545; $r^2 = 0.94$; Appendix Table A1) has been reported to significantly increase TNC protein plasma levels in patients carrying the C allele at rs7021589.[16] In our study, patients carrying the C allele at rs7021589 were protected against large recurrent defects.

Haplotype analysis revealed that in contrast to rs1138545, SNP rs2104772 (located in exon 17) is less influential but still significantly associated with a failure to heal (Table 6: haplotype 4). rs2104772 transcribes into the FNIII D domain of TNC protein (Figure 2). In contrast to the FNIII 5 domain, the FNIII D domain is found only in large isoforms (isoforms 1-5) of the TNC protein.[23] To understand the potential functionality of rs2104772, the role of large

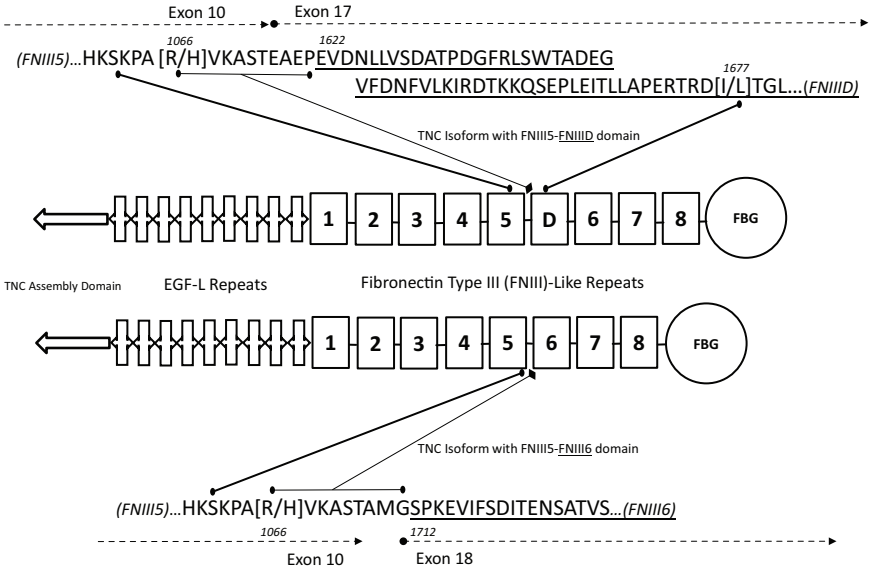

Figure 2. The depicted segment[23] of TNC–isoform 5 containing transcripts from exon 10 (FNIII 5) and exon 17 (FNIII D) shows that the physical distance of rs1138545 and rs2014772 is much shorter than what the position numbers in the ''canonical'' sequence (isoform 1) suggest. Of note, 23 amino acids upstream from rs2104772 lies the peptide sequence ($_{1646}$VFDNFVLK$_{1653}$), which binds integrin $\alpha7\beta1$ and thereby promotes the extension of neuronal processes.

isoforms of TNC protein must be put in the context of tendon healing. It is known that large TNC isoforms are required at times of active tissue repair, where they aid dynamic tissue organization through modulating cell proliferation (eg, fibroblasts/myofibroblasts), reduce cell adhesions, and allow cell migration through prevention of focal adhesion formation.[22] However, the short proadhesive TNC isoform containing the FNIII 5 domain but not the FNIII D domain (Figure 2) binds strongly to fibronectin fibrils, perlecan, periostin,[9] contactin, and other ECM molecules.[7] Therefore, the short TNC isoform meets requirements for the remodeling phase of tendon-to-bone healing[2] because it mediates stability of newly formed tissues and promotes cell attachment and the formation of focal adhesions, resulting in increased stiffness of the cellular environment through noncovalent matrix cross-linking.[22]

The association of rs2104772 found in our study is not unique but resonates with findings of other studies. According to Matsuda et al,[19] the A allele at rs2104772 might result in impaired structural stability of the FNIII D domain of the TNC protein; it is associated with asthma in Japanese adult patients; and it could affect the integrity of small airways during disease exacerbation. In children from the PARSIFAL study, rs2104772 was associated with chronic rhinoconjunctivitis.[25] In South African cohorts with Caucasian ancestry, rs2104772 was associated with chronic inflammation in Achilles tendons.[30] Patients carrying the A allele at rs2104772 were more susceptible to Achilles tendinopathy than healthy controls (who carried the T allele more frequently). Of note, rs2104772 is also part of a much larger cluster of risk SNPs for Achilles tendinitis, including ECM synthesis protein Col27A1 and cell signaling pathway proteins IL6, IL-1b, and CASP8,[29] which supports the notion of a polygenic model, meaning that tendon disorders may have associations with more than only 1 gene.

Limitations

The studied TNC region was rather small when compared with other musculoskeletal association studies where multiple genes or gene interactions were studied.[29,30] However, the 15 assessed SNPs represent the result of a much larger candidate gene search of risk factors for degenerative RCTs,[14] and we regard them as "hot spots" for failures to heal. Second, the described genetic associations are not necessarily causal to the observed phenotype. To determine the relevance of our findings further, we propose to test the functionality of *TNC* gene risk alleles. This could be done through binding assays of, for example, the TNC–FNIII 5 domain with various cytokines but also through tendocyte cell cultures with knockout sequences of TNC–FNIII 5 and TNC–FNIII D domains. It might well be worthy to look at the stability of TNC mRNA because, for example, in consideration of the findings within the translation of the *Col5A1* gene, the Achilles tendinopathy phenotype was linked to mRNA stability changes based on a single SNP in the *Col5A1* gene.[1] Third, we were not able to measure the local expression of TNC protein or TNC protein domains in postoperative tendons, which limits conclusions about the functional effects of our genetic findings. Also, despite the inclusion of most traditional covariates for recurrent rotator cuff defects, some potential covariates (tendon quality, osteoporosis, fatty degeneration of the rotator cuff) were not continuously assessed and are therefore missing in multivariate analyses. Last, the herein-reported genetic associations are

based on the assessment of the repaired rotator cuff at only 1 point in time postoperatively (sonography at 1 year) and hence do not allow for statements about potential genetic influences on the chronology of tendon healing.

One strength of our study is the minimal bias from the surgical technique, surgeons, and postoperative physiotherapy, because we used the same technique performed by 1 surgeon in all patients. Physiotherapy was standardized in our hospital but was performed in other facilities for <10% of patients. In addition, our cohort is very homogeneous, with all patients presenting with atraumatic initial tears and atraumatic recurrent defects in all cases.

Summary

Statistical comparisons of genotypes and allele frequencies among patients of the 3 outcome groups allowed us to define a risk-related combination of alleles (the "risk haplotype"). The 3-letter combination [CAG] thus means that patients carrying the C allele at rs1138545, the A allele at rs2104772, and the G allele at rs10759752 have a significantly increased risk to develop a large recurrent defect after rotator cuff repair.

CONCLUSION

Eight *TNC* SNPs, including 2 variants with potential functionality in tendon healing, are associated with a failure to heal. The respective gene segment is worthy of further investigation.

REFERENCES

1. Abrahams Y, Laguette MJ, Prince S, Collins M. Polymorphisms within the COL5A1 3′-UTR that alters mRNA structure and the MIR608 gene are associated with Achilles tendinopathy. *Ann Hum Genet*. 2013;77(3):204-214.
2. Bedi A, Maak T, Walsh C, et al. Cytokines in rotator cuff degeneration and repair. *J Shoulder Elbow Surg*. 2012;21(2):218-227.
3. Chaudhury S, Xia Z, Thakkar D, Hakimi O, Carr AJ. Gene expression profiles of changes underlying different-sized human rotator cuff tendon tears. *J Shoulder Elbow Surg*. 2016;25(10):1561-1570.
4. Chillemi C, Petrozza V, Garro L, et al. Rotator cuff re-tear or non-healing: histopathological aspects and predictive factors. *Knee Surg Sports Traumatol Arthrosc*. 2011;19(9):1588-1596.
5. Chung SW, Oh JH, Gong HS, Kim JY, Kim SH. Factors affecting rotator cuff healing after arthroscopic repair: osteoporosis as one of the independent risk factors. *Am J Sports Med*. 2011;39(10):2099-2107.
6. De Laporte L, Rice JJ, Tortelli F, Hubbell JA. Tenascin C promiscuously binds growth factors via its fifth fibronectin type III-like domain. *PLoS One*. 2013;8(4):e62076.
7. Giblin SP, Midwood KS. Tenascin-C: form versus function. *Cell Adh Migr*. 2015;9(1-2):48-82.
8. Iannotti JP, Deutsch A, Green A, et al. Time to failure after rotator cuff repair: a prospective imaging study. *J Bone Joint Surg Am*. 2013;95(11):965-971.
9. Kii I, Nishiyama T, Li M, et al. Incorporation of tenascin-C into the extracellular matrix by periostin underlies an extracellular meshwork architecture. *J Biol Chem*. 2010;285:2028-2039.
10. Killian ML, Cavinatto L, Shah SA, et al. The effects of chronic unloading and gap formation on tendon-to-bone healing in a rat model of massive rotator cuff tears. *J Orthop Res*. 2014;32(3):439-447.
11. Killian ML, Cavinatto LM, Ward SR, Havlioglu N, Thomopoulos S, Galatz LM. Chronic degeneration leads to poor healing of repaired massive rotator cuff tears in rats. *Am J Sports Med*. 2015; 43(10):2401-2410.
12. Kim HM, Caldwell JM, Buza JA, et al. Factors affecting satisfaction and shoulder function in patients with a recurrent rotator cuff tear. *J Bone Joint Surg Am*. 2014;96(2):106-112.
13. Kluger R, Bock P, Mittlböck M, Krampla W, Engel A. Long-term survivorship of rotator cuff repairs using ultrasound and magnetic resonance imaging analysis. *Am J Sports Med*. 2011;39(10):2071-2081.
14. Kluger R, Burgstaller J, Vogl C, Brem G, Skultety M, Mueller S. A candidate gene approach identifies six SNPs in tenascin-C (TNC) associated with degenerative rotator cuff tears [published online June 1, 2016]. *J Orthop Res*.
15. Le BT, Wu XL, Lam PH, Murrell GA. Factors predicting rotator cuff retears: an analysis of 1000 consecutive rotator cuff repairs. *Am J Sports Med*. 2014;42(5):1134-1142.
16. Lourdusamy A, Newhouse S, Lunnon K, et al; AddNeuroMed Consortium, Dobson R; Alzheimer's Disease Neuroimaging Initiative. Identification of cis-regulatory variation influencing protein abundance levels in human plasma. *Hum Mol Genet*. 2012;21(16):3719-3726.
17. Mall NA, Tanaka MJ, Choi LS, Paletta GA Jr. Factors affecting rotator cuff healing. *J Bone Joint Surg Am*. 2014;96(9):778-788.
18. Martino MM, Briquez PS, Güç E, et al. Growth factors engineered for super-affinity to the extracellular matrix enhance tissue healing. *Science*. 2014;343(6173):885-888.
19. Matsuda A, Hirota T, Akahoshi M, et al. Coding SNP in tenascin-C Fn-III-D domain associates with adult asthma. *Hum Mol Genet*. 2005;14:2779-2786.
20. McCarron JA, Derwin KA, Bey MJ, et al. Failure with continuity in rotator cuff repair ''healing.'' *Am J Sports Med*. 2013;41(1):134-141.
21. McElvany MD, McGoldrick E, Gee AO, Neradilek MB, Matsen FA 3rd. Rotator cuff repair: published evidence on factors associated with repair integrity and clinical outcome. *Am J Sports Med*. 2015;43(2): 491-500.
22. Midwood KS, Chiquet M, Tucker RP, Orend G. Tenascin-C at a glance. *J Cell Sci*. 2016;129(23):4321-4327.
23. NCBI Sequence Viewer. UniProtKB protein: TENA_HUMAN [accession P24821]. https://www.ncbi.nlm.nih.gov/projects/sviewer/. Accessed February 15, 2017.
24. Nho SJ, Shindle MK, Adler RS, Warren RF, Altchek DW, MacGillivray JD. Prospective analysis of arthroscopic rotator cuff repair: subgroup analysis. *J Shoulder Elbow Surg*. 2009;18(5):697-704.
25. Orsmark-Pietras C, Melén E, Vendelin J, et al; PARSIFAL Genetics Study Group. Biological and genetic interaction between tenascin C and neuropeptide S receptor 1 in allergic diseases. *Hum Mol Genet*. 2008;17(11):1673-1682.
26. Park JS, Park HJ, Kim SH, Oh JH. Prognostic factors affecting rotator cuff healing after arthroscopic repair in small to medium-sized tears. *Am J Sports Med*. 2015;43(10):2386-2392.
27. Robertson CM, Chen CT, Shindle MK, Cordasco FA, Rodeo SA, Warren RF. Failed healing of rotator cuff repair correlates with altered collagenase and gelatinase in supraspinatus and subscapularis tendons. *Am J Sports Med*. 2012;40(9):1993-2001.
28. Saccomanno MF, Sircana G, Cazzato G, Donati F, Randelli P, Milano G. Prognostic factors influencing the outcome of rotator cuff repair: a systematic review. *Knee Surg Sports Traumatol Arthrosc*. 2016; 24(12):3809-3819.
29. Saunders CJ, van der Merwe L, Cook J, Handley CJ, Collins M, September AV. Extracellular matrix proteins interact with cell-signaling pathways in modifying risk of Achilles tendinopathy. *J Orthop Res*. 2015;33(6):898-903.

30. Saunders CJ, van der Merwe L, Posthumus M, et al. Investigation of variants within the COL27A1 and TNC genes and Achilles tendinopathy in two populations. *J Orthop Res*. 2013;31(4):632-637.
31. Sinnwell JP, Schaid DJ. *Haplo.Stats: R Package* [computer software]. Version 1.5.4. 2011.
32. Tashjian RZ, Granger EK, Zhang Y, Teerlink CC, Cannon-Albright LA. Identification of a genetic variant associated with rotator cuff repair healing. *J Shoulder Elbow Surg*. 2016;25(6):865-872.
33. Udalova IA, Ruhmann M, Thomson SJ, Midwood KS. Expression and immune function of tenascin-C. *Crit Rev Immunol*. 2011;31(2):115-145.
34. Warnes G, Gorjanc G, Leisch F, et al. *Genetics: R Package* [computer software]. Version 1.3.6. 2011.
35. Yamamoto A, Takagishi K, Osawa T, et al. Prevalence and risk factors of a rotator cuff tear in the general population. *J Shoulder Elbow Surg*. 2010;19(1):116-120.