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Polymorphic variants in tenascin-C (*TNC*) are associated with atherosclerosis and coronary artery disease

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Abstract

Tenascin-C (*TNC*) is an extracellular matrix protein implicated in biological processes important for atherosclerotic plaque development and progression, including smooth muscle cell migration and proliferation. Previously, we observed differential expression of *TNC* in atherosclerotic aortas compared with healthy aortas. The goal of this study was to investigate whether common genetic variation within *TNC* is associated with risk of atherosclerosis and coronary artery disease (CAD) in three independent datasets. We genotyped 35 single nucleotide polymorphisms (SNPs), including 21 haplotype tagging SNPs, in two of these datasets: human aorta tissue samples ($n = 205$) and the CATHGEN cardiovascular study ($n = 1,325$). Eleven of these 35 SNPs were then genotyped in a third dataset, the GENECARD family study of early-onset CAD ($n = 879$ families). Three SNPs representing a block of linkage disequilibrium, rs3789875, rs12347433, and rs4552883, were significantly associated with atherosclerosis in multiple datasets and demonstrated consistent, but suggestive, genetic effects in all analyses. In combined analysis rs3789875 and rs12347433 were statistically significant after Bonferroni correction for 35 comparisons, $p = 2 \times 10^{-6}$ and 5×10^{-6} , respectively. The SNP rs12347433 is a synonymous coding SNP and may be biologically relevant to the mechanism by which tenascin-C influences the pathophysiology of CAD and atherosclerosis. This is the first report of genetic association between polymorphisms in *TNC* and atherosclerosis or CAD.

Introduction

Atherosclerosis and atherothrombotic diseases, including coronary artery disease (CAD), are the leading cause of death in the United States and are projected to remain so through 2020 (Rosamond et al. 2008). Atherosclerosis is a process that results in the focal deposition of lipids in the interior surface of blood vessels, and over time these deposits can develop into atherosclerotic plaques, which contain a lipid core, inflammatory matter like macro-phages, and smooth muscle cells (Watkins and Farrall 2006). In addition to known environmental risk factors, such as diet and exercise, family history has long been recognized as an independent CAD risk-factor even after adjusting for the effect of shared environment (Shea et al. 1984; Vaidya et al. 2007). Through the study of Mendelian forms of atherosclerosis, we have gained many insights into the molecular mechanisms of plaque formation (reviewed in Lusis et al. 2004; Watkins and Farrall 2006). Yet the genes implicated thus far in these disorders are thought to account for only a fraction of the total genetic risk for more common forms of CAD. One locus on chromosome 9p21.3 has been consistently associated with common, complex CAD in multiple datasets (Helgadottir et al. 2007; McPherson et al. 2007; Wellcome Trust Case Control Consortium, 2007; Samani et al. 2007), but like the Mendelian variants this locus is also thought to account for only a fraction of total CAD genetic risk (Schunkert et al. 2008), implying that other CAD genetic factors remain to be identified.

Our group has successfully used genomic convergence (Hauser et al. 2003a) to identify novel candidate genes implicated in the development of CAD (Connelly et al. 2006, 2008; Wang et al. 2007, 2008; Sutton et al. 2008; Shah et al. 2009). The approach employs multiple lines of evidence, such as linkage, association, and gene expression, to implicate a gene or genetic variant in a disease phenotype. In 2004, we identified 208 genes whose expression patterns could distinguish between severely and minimally diseased human aorta samples (Seo et al. 2004). When these genes were ranked in order of predictive power, the 33rd gene on the list was tenascin-C (*TNC*; MIM 187380), also known as hexabrachion. Tenascin-C is a glycoprotein component of the extracellular matrix (ECM) that has been implicated in a variety of cellular processes relevant to atherosclerosis, including cell proliferation, migration, and apoptosis (reviewed in Jones and Jones 2000a, b). Expression of tenascin-C is tightly controlled in adults and is upregulated in tissues undergoing wound healing, such as the myocardium following a myocardial infarction (MI) (Imanaka-Yoshida et al. 2001a). There is a positive correlation between tenascin-C expression and advanced stages of atherosclerotic plaque development, specifically with the degree of inflammation present in an atherosclerotic plaque (Wallner et al. 1999) and with ruptured plaques in patients with acute coronary syndromes (Kajiwara et al. 2004). Tenascin-C also plays an integral role in neointimal hyperplasia (Yamamoto et al. 2005; Sawada et al. 2007) by promoting the migration (LaFleur et al. 1997) and proliferation (Sharifi et al. 1992) of arterial smooth muscle cells (SMCs). Additional evidence comes from animal models, as *TNC* falls within a quantitative trait locus (*Ath8*) that influences the difference in susceptibility to atherosclerosis between the NZB/BINJ and SM/J mouse strains (Pitman et al. 1998).

Given that tenascin-C plays an important role in the atherosclerotic process and is differentially expressed in human atherosclerotic aortas, we hypothesized that genetic variants within *TNC* may influence its function and modulate risk for atherosclerotic diseases. We sought to characterize the effect of common single nucleotide polymorphisms (SNPs) within the *TNC* locus on atherosclerotic disease risk in three atherosclerosis/CAD studies and found three SNPs in high linkage disequilibrium (LD) that were associated with increased disease risk across all three datasets. To our knowledge, this is the first report of association between *TNC* SNPs and the atherosclerotic/ CAD phenotypes.

Materials and methods

Human atherosclerotic aorta samples and *TNC* expression

Human aorta samples were collected from heart transplant donors as previously described (Seo et al. 2004). Briefly, DNA ($n = 205$) and RNA ($n = 104$) were isolated from aorta tissue samples and used in genetic association studies or gene expression analysis, respectively. Thirty aortas had samples taken from both proximal and distal portions of the aorta. Gene expression levels of *TNC* in the aorta samples were measured using the tag 32818_at on the Affymetrix GeneChip U95Av2 array (Affymetrix, Santa Clara, CA, USA). Expression signal intensity values were $\log_2$ transformed then normalized using quantile normalization.

In addition to DNA and RNA isolation, the aortas were also scored for the presence of atherosclerosis using two different measures of disease. Early atherosclerotic lesions were measured by quantifying the proportion of the aorta that was positive for Sudan IV staining using imaging processing methods. Advanced atherosclerotic lesions were detected by quantifying the proportion of the aorta with raised lesions using methodologies from the Pathobiological Determinants of Atherosclerosis in Youth (PDAY) study (Cornhill et al. 1985). Because the PDAY study found that disease burden shows sagittal symmetry across the aorta, mirroring tissue sections could be used to correlate the atherosclerotic disease phenotype on one side of the aorta with the gene expression level and SNP genotype of the

opposite side of the aorta. The presence of any raised lesion area in an aorta defined a case for the aorta logistic regression analysis described below. The limited clinical characteristics corresponding to the tissue samples are shown in Table 1.

CATHGEN CAD case-control samples

The collection of CATHeterization GENetics (CATHGEN) case-control subjects has been described elsewhere (Connelly et al. 2006, 2008; Wang et al. 2007, 2008; Sutton et al. 2008; Shah et al. 2009). Briefly, CATHGEN study participants were recruited through the cardiac catheterization laboratories at Duke University Hospital (Durham, NC, USA), with approval from the Duke Institutional Review Board, and all participants signed informed consent. Cases and controls were selected based on extent of CAD as measured by the CAD index (CADi), a numerical summary of coronary angiographic data (Smith et al. 1991) that has been shown to be an excellent predictor of clinical outcome (Kong et al. 2002). CATHGEN cases had a CADi ≥ 32 , which is the equivalent of at least a 95% stenosis in at least one major epicardial vessel. For patients above 55 years of age at disease onset (older affecteds, OA), a higher CADi threshold (≥ 74 , equivalent to three vessel disease with at least a 95% stenosis in the proximal left anterior descending coronary artery) was used to account for the higher baseline extent of CAD in this group. Subjects with 75% or greater stenosis in the left main coronary artery were defined as left main (LM) cases regardless of age of CAD onset. Controls had an age at catheterization ≤ 60 years and had no CAD as defined by coronary angiography and no documented history of MI, heart transplant, peripheral or cerebrovascular disease, or interventional or surgical coronary revascularization procedures. Although these controls may not be representative of population-based controls, because they presented for cardiac catheterization, the angiography demonstrated that these individuals were free of CAD and can therefore serve as excellent controls for our analyses.

Although the CATHGEN study participants ($n = 920$ cases and 405 controls) comprised a variety of ethnic groups, the majority of subjects were Caucasian ($n = 695$ cases and 289 controls). To avoid the confounding effects of population stratification while maximizing our power to detect genetic associations, we chose to analyze the Caucasian participants only. The clinical characteristics of the Caucasian CATHGEN subjects are presented in Table 1.

GENECARD early-onset CAD proband samples

The collection of the Genetics of Early Onset Cardiovascular Disease (GENECARD) family-based CAD subjects has been described in detail elsewhere (Hauser et al. 2003b). Families were recruited over a four-year period at six international sites, and all participants signed a consent form approved by the responsible institutional review board or local ethics committee. Qualified participants were required to have at least one of the following: MI or unstable angina (acute coronary syndrome), coronary catheterization demonstrating significant disease (at least a 50% stenosis in one major epicardial vessel), interventional coronary revascularization procedure (percutaneous transluminal coronary intervention or coronary artery bypass grafting), or a functional test documenting reversible myocardial ischemia with cardiac imaging. The qualifying event or procedure must have occurred ≥ 51 years in men or ≥ 56 years in women. For families to be eligible for inclusion in the study, they were required to include at least two siblings, each of whom met the above-described diagnostic criteria and were available for sampling and data collection.

For our association analyses, the proband from each family was used in association testing against the CATH-GEN controls ($n = 759$). Because the majority of these probands are Caucasian ($n = 650$), we again chose to analyze associations only within the Caucasian probands to minimize confounding due to population stratification. The clinical

characteristics of the Caucasian GENECARD probands are presented in Table 1. As an alternative statistical approach, we also performed family-based association in the GENECARD affected-sibling pair families ($n = 879$ families). The clinical characteristics of the entire GENECARD family dataset have been published elsewhere (Hauser et al. 2003b; Connelly et al. 2006).

Haplotype tagging SNP selection and genotyping

A minimal set of haplotype tagging SNPs (htSNPs) covering the *TNC* gene, 5 kb upstream of the proximal promoter, and 5 kb downstream of the 3' untranslated region were selected using the Tagger pair-wise algorithm (de Bakker et al. 2005) on the HapMap website (version B35) to capture the predicted LD structure of *TNC*. To select htSNPs, we used a minor allele frequency (MAF) $\geq 5\%$ and $r^2 \geq 0.8$ in the CEU (μ Humain [CEPH] Utah residents with ancestry from northern and western Europe) HapMap population. For some LD blocks we genotyped additional SNPs to capture additional genetic variation or functional relevance.

Genomic DNA from whole blood or from aorta tissue was extracted and purified using the PureGene system (Gentra Systems, Minneapolis, MN, USA). Twenty-seven SNPs were genotyped using TaqMan allelic discrimination assays (Applied Biosystems, Foster City, CA, USA) in 384-well plate format. Quality control (QC) samples, composed of 12 reference genotype controls, two CEU pedigree individuals, and one no-template sample, were included in each quadrant of each 384-well plate. The QC samples were used to provide duplicate samples within one quadrant, across quadrants within one plate, and across plates. Genotype data for eight SNPs were generated in the CATHGEN dataset with part of a custom 1,536 oligo pool assay (OPA) on the Bead Station 500G SNP genotyping station (Illumina, San Diego, CA, USA) as part of an ongoing study. Each array included four QC samples, two CEPH pedigree individuals, and two identical in-plate controls. Results of the CEPH and QC sample genotyping were compared in order to identify possible sample plating errors and genotype-calling inconsistencies. Genotype call rates were above 95% for all SNPs. All SNPs were tested for significant deviations (p value < 0.001) from Hardy–Weinberg equilibrium in the Caucasian CATHGEN cases and controls with a χ^2 test, and no such deviations were observed (data not shown).

Statistical analyses

LD between pairs of SNPs was assessed using the Graphical Overview of Linkage Disequilibrium (GOLD) package (Abecasis and Cookson 2000) and visualized using the Haploview program (Barrett et al. 2005). All statistical analyses were performed using SAS 9.1 (SAS Institute Inc., Cary, NC, USA). To detect associations between *TNC* SNPs and expression levels in our aorta sample dataset, we used a mixed model as implemented in the PROC MIXED procedure in SAS to account for the fact that some aortas had samples taken from both proximal and distal portions of the aorta. The expression level of the *TNC* Affymetrix U95Av2 tag (32818_at) was modeled adjusting for age, sex, and race of the tissue donor.

Genotypic association in the CATHGEN case/control dataset and GENECARD probands was examined using two multivariable logistic regression models. Genotypes were coded as 0, 1, or 2 copies of the minor allele, i.e., a standard additive genetic model. The basic model adjusted for sex, while the full model adjusted for sex and known CAD risk factors (history of hypertension, history of diabetes mellitus, history of dyslipidemia, body mass index, and smoking history) as covariates. These adjustments hypothetically allow us to control for competing genetic pathways that are independent risk factors for CAD, thereby allowing us to detect a separate effect of genes on CAD risk. Genotypic associations with disease status

as defined by the presence of raised lesions in the aorta tissue samples were examined using three different multivariable logistic regression models. The three nested logistic models included the following independent variables: (1) SNP genotype, sex, race, and age; (2) *TNC* expression level, SNP genotype, sex, race, and age; and (3) *TNC* expression level, SNP genotype, interaction between expression level and genotype, sex, race, and age. In addition to the multivariable logistic regression, we also performed family based association in the entire GENECARD family dataset using the association in the presence of linkage (APL) test (Martin et al. 2003; Chung et al. 2007). This test appropriately accounts for the non-independence of affected siblings and is robust to population stratification.

Because the measures of CAD and atherosclerosis are different in each dataset and the anticipated effects and effect sizes are likely to be different in the three datasets, we created a summary measure of evidence using Fisher's formula to combine *p* values for heterogeneous datasets (Won et al. 2009). For this meta-analysis, we selected the most severe or advanced measures of atherosclerosis in each dataset and combined the *p* values from those tests: older affecteds from CATHGEN, family-based association using the APL test from GENECARD, and raised lesions from the aorta samples. Raw, uncorrected *p* values from each association test are presented in this report, and SNPs that pass the traditional *p* value <0.05 cutoff are considered statistically significant. When appropriate, results interpreted in the context of a conservative Bonferroni correction for 35 SNPs ($0.05/35 \approx 0.0014$) are also presented. In addition to the conservative Bonferroni multiple testing correction, we relied on the replication of results across multiple, independent datasets to validate associations and to minimize the possibility of false-positive findings.

Study design

Figure 1 represents the design of the study described here. The figure includes a description of the numerous datasets and statistical analyses performed.

Results

SNP selection and LD relationships

A total of 35 SNPs were genotyped across the *TNC* locus (Table 2; Fig. 2). Twenty-one haplotype tagging SNPs (htSNPs) were used to capture common genetic variation and 14 additional SNPs were selected either to capture functional relevance within associated blocks of linkage disequilibrium (LD) or to cover additional genetic variation within the gene. Three SNPs (rs13321, rs2104772, and rs1757095) were non-synonymous; two SNPs (rs12347433 and rs1061495) were synonymous; and the remaining 30 SNPs were located in either intronic, flanking, or intergenic yet neighboring regions of the *TNC* locus. The LD patterns were similar for CATHGEN cases and controls (data not shown). The LD plot for the CATHGEN controls is shown in Fig. 3. As expected from the selection criteria used to select htSNPs in the CEU HapMap samples, the majority of genotyped SNPs were not highly correlated ($r^2 < 0.8$). Therefore, we believe we captured most of the common genetic variation across the *TNC* locus.

Single SNP marker association in human aorta sample dataset

The 205 aorta samples were stratified into two groups of cases and controls based on the presence of atherosclerosis using two measures of disease: Sudan IV staining (early atherosclerosis) and presence of raised lesions (late atherosclerosis). No significant association was detected between any *TNC* SNP and Sudan IV staining (data not shown). However, 12 SNPs were significantly associated with the presence of raised lesions ($p = 0.001$ – 0.05 , Table 3). The three most strongly associated SNPs, rs4452883, rs12347433, and

rs3789875 (odds ratios [OR] = 3.4–4.5, Table 3), were in high LD ($r^2 > 0.94$) in both Caucasian HapMap samples and the CATHGEN CAD dataset (Fig. 3).

Previously, we identified differential expression of *TNC* in diseased aortas (Seo et al. 2004). We next investigated whether any of the 35 SNPs were associated with differential expression of *TNC*. Because RNA was only extracted from 104 aorta samples, our gene expression dataset was smaller than our genotyping dataset. We did not detect any significant associations between SNP genotypes and *TNC* expression level in the entire aorta dataset or in the aortas with raised lesions (data not shown). We detected association between SNP genotype and presence of raised lesions after controlling for sex, age, and race for 16 SNPs ($p = 0.0003$ – 0.04). When we also included *TNC* gene expression level, the relationship between SNP genotype and raised lesions remained significant for 14 SNPs ($p = 0.0032$ – 0.03). Finally, when adding in the interaction between gene expression and genotype, the interaction term was significant for four SNPs ($p = 0.01$ – 0.05). The genotypes of the three SNPs most strongly associated with raised lesions, rs4452883, rs3789875, and rs12347433, were significant across all three of these models. The interaction between SNP genotype and gene expression was significant for rs4452883 and rs3789875 ($p = 0.023$ and 0.048 , respectively) but was only marginally significant for rs12347433 ($p = 0.054$).

Single SNP marker association in the CATHGEN CAD case–control dataset

We then sought to determine whether the SNP associations observed in the aorta samples could be detected in atherosclerotic phenotypes in larger datasets. We genotyped the same set of 35 *TNC* SNPs across the CATHGEN dataset. Three SNPs (rs1999223, rs12347433, and rs944225) were significantly associated with CAD in both the basic and full models ($p = 0.03$ – 0.05 , Table 4; Fig. 4).

Only one of the significant SNPs from the aorta raised lesion analysis, rs12347433, was significant in the comparison of CATHGEN cases to controls ($p = 0.04$ basic, 0.03 full; OR = 1.3). The other two SNPs associated in the aorta dataset also showed consistent ORs for the minor allele (as defined in the overall CATHGEN dataset, including both cases and controls), but the ORs were not significantly different from 1.0 in the full model that included all CATHGEN cases (Table 4).

To investigate the genetic associations further in a priori specified analyses, we stratified CATHGEN cases by age of disease onset (with >55 years defined as “older affected” [OA] cases) as a proxy for disease severity, since these cases must meet a higher threshold of disease to be considered affected (see “Materials and methods”). The OA cases produced a similar pattern of genetic association as in the entire CATHGEN dataset and in the raised lesion aorta samples, but with stronger association despite the smaller sample sizes ($p = 0.001$ – 0.04 , Table 4). Using both models, four SNPs were significantly associated with CAD in the OA cases ($p = 0.001$ – 0.04) including the three most significant SNPs from the aorta raised lesion analysis. Furthermore, the ORs for these three SNPs showed the same direction of effect as in the aorta analysis (ORs = 1.53–1.69, Table 4).

Our results in the raised lesion aortas and CATHGEN OA cases led us to hypothesize that polymorphisms in *TNC* are associated with severe forms of atherosclerosis and CAD. As an additional test of our hypothesis, we utilized the angiographic data to identify a subset of cases ($n = 244$) that have 75% or greater stenosis in the left main coronary artery. Left main coronary artery disease (LM) is considered to be a severe form of CAD owing to its poor clinical outcome (Kang et al. 2009) and is thought to have a stronger genetic component than CAD that manifests in more peripheral coronary arteries (Fischer et al. 2005). As shown in Table 4, five SNPs were significantly associated with CAD in LM cases compared

with the no CAD controls ($p = 0.004$ – 0.03 , ORs = 1.08–2.20), including the three SNPs that were significantly associated in the aorta raised lesion and CATHGEN OA analyses.

Single SNP marker association in the GENECARD CAD family-based dataset

For further replication, we investigated a third dataset comprising families enrolled in the GENECARD study of early-onset CAD (Hauser et al. 2003b), analyzing both the probands compared with CATHGEN controls and then the GENECARD families for family-based association. Only 11 of the 35 *TNC* SNPs were genotyped within the GENECARD subjects, because they either represented an LD block or were significant in at least one dataset or sub-analysis of a dataset. Using both basic and full models in the analysis of the probands, we detected three significant SNPs using the basic model ($p = 0.02$ – 0.04), including two of the SNPs (rs3789875 and rs12347433) that were significant in the aortas and CATHGEN data-sets. The ORs for rs3789875 and rs12347433 showed the same direction of effect as in the aorta and CATHGEN datasets, implying the minor allele confers risk for CAD. The three SNPs significant in the basic model did not achieve statistical significance ($p < 0.05$) under the full model ($p = 0.06$ – 0.09); however, the ORs continued to be consistent with the results from the aorta and CATHGEN datasets. Family-based association for early-onset CAD using APL showed modest results in support of *TNC*, producing the lowest p values for SNPs rs3789875 and rs12347433 ($p = 0.05$ and 0.08 , respectively) (Table 4). A graphical summary of all genetic associations across all three datasets and selected sub-analyses within the aorta and CATHGEN datasets is presented in Fig. 4.

A meta-analysis of SNPs genotyped across all three independent datasets ($n = 11$ SNPs) identified two SNPs with consistent and significant evidence for association with severe disease: rs3789875 and rs12347433, with p values of 0.000002 and 0.000005, respectively. These SNPs are identified in Fig. 4.

Discussion

The influence of genetic polymorphisms in *TNC* on human health and disease has not been well studied. The few studies examining *TNC* polymorphisms have linked an intronic GT dinucleotide repeat to increased risk for Achilles tendon injuries (Mokone et al. 2005), a coding SNP to adult asthma (Matsuda et al. 2005), and several coding and noncoding SNPs to several childhood asthma/ allergy phenotypes (Orsmark-Pietras et al. 2008). In addition to these small genetic variations, a copy number increase across the *TNC* locus was identified in several patients with pediatric ependymomas (Puget et al. 2009). Yet to our knowledge, our study represents the first comprehensive analysis of common genetic variation within the *TNC* gene in the context of atherosclerosis and is the first to identify *TNC* genetic association with increased risk of CAD and atherosclerosis.

Adding to previous work by Seo et al. (2004) showing differential expression in atherosclerotic human aortas relative to healthy aortas, we observed three SNPs within the same LD block in *TNC* that were associated with atherosclerotic plaques in aortas and with CAD in two independently ascertained CAD patient datasets. Genetic associations were strongest in these datasets when we used a priori stratified analyses that examined clinically severe or advanced forms of CAD and atherosclerosis. The CATHGEN OA cases had three vessel disease with at least a 95% stenosis in the proximal left anterior descending coronary artery; the CATHGEN LM cases had left main artery disease, which is associated with a poor clinical prognosis (Kang et al. 2009); and the GENECARD pro-bands had early-onset CAD, which by nature of the age of affected individuals may be considered a more “aggressive” form of CAD than typically encountered.

While the *p* values presented in this paper have not been corrected for multiple testing comparisons due to ongoing debate regarding which method is the most appropriate correction (Rice et al. 2008), we have relied on the replication of significant results across three independently ascertained patient datasets to reduce the probability of false positive findings. However, if we were to implement a conservative Bonferroni correction for the 35 SNPs we genotyped across TNC ($0.05/35 \approx 0.001$), then both rs4452883 and rs12347433 would pass this stringent threshold in the aorta raised lesion and CATHGEN OA analyses, respectively, as well as in the combined meta-analysis, thus suggesting consistent, independent support for association of genetic variation in *TNC* with atherosclerosis.

Genetic association in *TNC* was localized to an LD block that contains rs12347433, a putative causal SNP located in exon 22. Although rs12347433 is a synonymous SNP, producing no coding change in the final tenascin-C protein product, its location translates to the fibronectin type III-8 domain in the tenascin-C protein, which is found in all *TNC* transcripts (Pas et al. 2006). Synonymous polymorphisms are increasingly being recognized as having the potential to produce phenotypic variation even though they do not encode an amino acid change in the final protein product. Various mechanisms, including alterations in mRNA structure and stability caused by changes in codon usage and translational pausing (Kimchi-Safarty et al. 2007; Nackley et al. 2006), or alterations in splicing efficiency caused by changes in exonic splicing enhancer or silencer sequences (Nielsen et al. 2007) have been mechanisms postulated for the influence of synonymous SNPs on a disease phenotype. Such mechanisms would be consistent with our lack of observed association between rs12347433 and gene expression in the aortas. However, further functional work will be needed to characterize the mechanism(s) through which rs12347433 may be influencing CAD disease risk.

When we examined association between *TNC* SNPs and atherosclerotic phenotypes in aortic tissue, we observed significant association with the presence of raised lesions but not with Sudan IV staining. This association is consistent with previous reports that have detected tenascin-C in both human and mouse atherosclerotic plaques, particularly those in the later stages of plaque development with high amounts of macrophage infiltrate (Wallner et al. 1999; Kajiwarra et al. 2004; von Lukowicz et al. 2007; Pedretti et al. 2009). One possible explanation for this association is that lesions with a high degree of macrophage infiltrate are highly prone to rupturing (van der Wal et al. 1994). Tenascin-C may destabilize atherosclerotic plaques and make them more prone to rupture through its upregulation of matrix metalloproteinases (MMPs) (Wallner et al. 2004), proteins thought to play an important role in plaque rupture (Shah and Galis 2001). However, further studies would be required to test this hypothesis.

In addition to merely being expressed in advanced atherosclerotic plaques, tenascin-C also appears to play an important role in the formation and progression of these plaques. A series of experiments in mice with coronary artery bypass grafting-induced stenosis showed that following injury to the arterial wall, tenascin-C expression transiently increases (Imanaka-Yoshida et al. 2001b), and that the suppression of this expression, either with the compound cilostazol or in a *TNC* knock-out mouse, results in reduced neointimal hyperplasia (Fujinaga et al. 2004; Yamamoto et al. 2005; Sawada et al. 2007). The same increase in expression following arterial wall injury has been replicated using rats with balloon catheter-induced arterial injury (Hedin et al. 1991). On a cellular level, the suppression of tenascin-C expression is associated with reduced neointimal cell proliferation (Yamamoto et al. 2005), while expression of tenascin-C is correlated with SMC apoptosis (Wallner et al. 2004), SMC proliferation (Sharifi et al. 1992), and with a change in SMC phenotype from a non-proliferative “contractile” phenotype to a migratory “synthetic” state (Hedin et al. 1991), a key feature of SMC migration (Doran et al. 2008). Tenascin-C inhibits the attachment of

both rat and human aortic SMCs to fibronectin (LaFleur et al. 1994), implying that the presence of tenascin-C in the ECM has a destabilizing effect on cell-ECM interactions, possibly explaining the mechanism by which tenascin-C could lead to increased cell migration. The proliferation, migration, and apoptotic death of SMCs are all key features found in later stages of atherosclerotic plaque development (Watkins and Farrall 2006).

Key to SMC migration and proliferation during plaque development are changes to the SMC actin cytoskeleton mediated through the Rho-GTPase pathway (Rolfe et al. 2005). Several reports suggest that tenascin-C interacts with various proteins in this pathway to help promote SMC migration and proliferation, although the literature is conflicting as to whether tenascin-C is an upstream effector (Wenk et al. 2000; Midwood and Schwarzbauer 2002; Laufs et al. 1999; Ili et al. 1995) or a downstream target (Chapados et al. 2006; McKean et al. 2003). We have previously observed strong evidence for genetic linkage association to several genes in the Rho-GTPase pathway on chromosome 3q13, including *KALRN*, *CDGAP*, and *MYLK* (Hauser et al. 2004; Wang et al. 2007) in our GENCARD family study. Although further work is needed to clarify how tenascin-C fits into this pathway, its interaction with the Rho-GTPase pathway may offer a functional mechanism by which tenascin-C could promote the SMC migration and proliferation crucial to atherosclerotic plaque development. Based on our findings of a consistent association between *TNC* polymorphisms and atherosclerosis along with the reports from the literature, we hypothesize that tenascin-C is an important factor in the later stages of atherosclerotic plaque formation, because of its roles in promoting SMC migration and proliferation and its replicated association with severe and advanced atherosclerotic and CAD phenotypes.

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Fig. 1. Summary of overall study design employed

Fig. 2. *TNC* gene schematic and genotyped SNPs. *TNC* gene schematic showing the 35 SNPs genotyped across the *TNC* locus according to their physical location. Non-synonymous SNPs are denoted by a *double solid arrow*, synonymous SNPs are denoted by a *double open arrow*, intronic SNPs are denoted by a *single solid arrow*, and intergenic SNPs are denoted by a *single open arrow*. The *TNC* gene is located on the reverse strand of the reference assembly, and is hence drawn from 3' to 5' to reflect its physical orientation

Fig. 3. LD relationships between genotyped *TNC* SNPs. LD relationships (r^2 values) between genotyped SNPs are shown for CATHGEN Caucasian controls. The LD plot was visualized with the Haploview program (Barrett et al. 2005). The shading of the blocks indicates the strength of the LD relationship, with *black blocks* showing $r^2 = 1.0$ and *white blocks* showing $r^2 = 0.0$. The three most significant SNPs reported in this paper are highlighted by *black boxes* in the figure

Fig. 4. Graphical overview of *TNC* SNP associations. Graph showing the physical map location of each *TNC* SNP plotted against the $-\log_{10}(p\text{ value})$ for each SNP in each analysis (see text for details). Data from the full model in CATHGEN and GENE CARD probands are presented. Points above the horizontal dotted lines are significant at a threshold of $p = 0.05$ or 0.01. The *gray, dashed, vertical lines* highlight the p values for rs3789875 and rs12347433, the two significant SNPs from the meta-analysis

Table 1
Demographic characteristics of three CAD and atherosclerosis datasets

Characteristics	Aorta samples	Unaffected CATHGEN controls	All CATHGEN CAD cases	Older affected CATHGEN CAD cases	Left Main CATHGEN CAD cases	GENECARD probands
Total number	205	289	695	230	244	650
Age of onset or age at exam (mean $\pm$ SD)	36 $\pm$ 15	N/A	53 $\pm$ 11	66 $\pm$ 8	58 $\pm$ 12	44 $\pm$ 6
Age at exam (mean $\pm$ SD)	N/A	69 $\pm$ 7	59 $\pm$ 12	72 $\pm$ 8	67 $\pm$ 10	51 $\pm$ 7
Race (%): Caucasian	80	100	100	100	100	100
Asian	2	0	0	0	0	0
African American	12	0	0	0	0	0
Hispanic	6	0	0	0	0	0
Male (%)	61	43	82	76	81	71
Family history of CAD (%)	N/A	28	54	46	52	100
Body mass index, kg/m ² (mean $\pm$ SD)	N/A	28 $\pm$ 6	30 $\pm$ 6	28 $\pm$ 6	29 $\pm$ 6	31 $\pm$ 7
History of smoking (%)	N/A	39	65	52	59	26
History of diabetes (%)	N/A	15	31	30	29	23
History of hypertension (%)	N/A	66	68	74	74	61
History of myocardial infarction (%)	N/A	0	47	38	42	64
History of coronary artery bypass graft (%)	N/A	0	48	53	60	50
Systolic blood pressure, mmHg (mean $\pm$ SD)	N/A	149 $\pm$ 23	142 $\pm$ 24	149 $\pm$ 26	147 $\pm$ 26	126 $\pm$ 18
Diastolic blood pressure, mmHg (mean $\pm$ SD)	N/A	76 $\pm$ 13	76 $\pm$ 13	77 $\pm$ 12	77 $\pm$ 13	75 $\pm$ 12
Total cholesterol, mg/dL (mean $\pm$ SD)	N/A	189 $\pm$ 43	187 $\pm$ 54	173 $\pm$ 43	176 $\pm$ 39	219 $\pm$ 56
Low-density lipoprotein, mg/dL (mean $\pm$ SD)	N/A	105 $\pm$ 35	104 $\pm$ 40	99 $\pm$ 36	97 $\pm$ 31	144 $\pm$ 78
High-density lipoprotein, mg/dL (mean $\pm$ SD)	N/A	51 $\pm$ 19	40 $\pm$ 12	43 $\pm$ 13	42 $\pm$ 13	44 $\pm$ 55
Triglycerides, mg/dL (mean $\pm$ SD)	N/A	169 $\pm$ 133	225 $\pm$ 248	173 $\pm$ 98	194 $\pm$ 135	243 $\pm$ 319

Clinical characteristics are listed for the aorta tissue samples' donors, Caucasian CATHGEN controls and cases (including subgroups of the CATHGEN cases), and Caucasian GENECARD probands

N/A not available

Table 2
Genomic locations and minor allele frequencies of SNPs genotyped across the *TNC* locus

SNP locations		Minor allele frequencies										
SNP	Location (bp)	Gene location	Controls		Older affected cases		Left main cases		GENECARD probands		Aorta samples	
			Minor allele	MAF	Minor allele	MAF	Minor allele	MAF	Minor allele	MAF	Minor allele	MAF
rs10817691	116760083	3' of gene	C	0.476	A	0.496	A	0.494	N/A	N/A	C	0.459
rs873211	116775766	3' of gene	C	0.149	C	0.161	C	0.162	N/A	N/A	C	0.109
rs4978617	116804670	3' of gene	C	0.286	C	0.357	C	0.313	C	0.299	C	0.317
rs17239726	116811333	3' of gene	A	0.167	A	0.204	A	0.218	A	0.186	A	0.171
rs1999223	116815151	3' of gene	T	0.252	T	0.218	T	0.221	N/A	N/A	T	0.215
rs12156617	116818164	3' of gene	T	0.051	T	0.069	T	0.063	N/A	N/A	T	0.047
rs10117421	116818190	3' of gene	C	0.154	C	0.125	C	0.115	N/A	N/A	C	0.121
rs4452883	116819395	3' of gene	A	0.244	A	0.304	A	0.298	N/A	N/A	A	0.272
rs12555312	116819475	3' of gene	C	0.111	C	0.065	C	0.059	C	0.076	C	0.082
rs11787664	116821477	3' flanking gene	T	0.497	C	0.476	C	0.481	N/A	N/A	T	0.494
rs7035322	116826994	Intron 26	A	0.279	A	0.266	A	0.287	N/A	N/A	A	0.287
rs13321	116832404	Exon 24 (Q2008E)	C	0.284	C	0.28	C	0.293	C	0.281	C	0.282
rs3789875	116835109	Intron 22	A	0.242	A	0.304	A	0.301	A	0.278	A	0.279
rs12347433	116837418	Exon 22 (R1891R)	C	0.236	C	0.313	C	0.305	C	0.279	C	0.287
rs10759752	116843541	Intron 18	G	0.443	G	0.387	G	0.389	N/A	N/A	G	0.418
rs1061495	116844365	Exon 18 (T1734T)	G	0.233	G	0.27	G	0.27	N/A	N/A	G	0.217
rs2104772	116848606	Exon 17 (I1677L)	T	0.433	T	0.459	T	0.455	N/A	N/A	T	0.474
rs1547692	116852166	Intron 15	A	0.413	A	0.368	A	0.366	N/A	N/A	A	0.376
rs11789200	116852612	Intron 15	G	0.143	G	0.17	G	0.181	N/A	N/A	G	0.145
rs1060545	116861439	Intron 14	C	0.486	C	0.489	A	0.494	N/A	N/A	C	0.471
rs944225	116863722	Intron 13	C	0.253	C	0.232	C	0.243	C	0.227	C	0.25
rs16932160	116869808	Intron 10	A	0.358	A	0.33	A	0.336	A	0.342	A	0.332
rs955450	116872927	Intron 10	G	0.263	G	0.257	G	0.258	N/A	N/A	G	0.262
rs3789873	116875035	Intron 10	C	0.266	C	0.265	C	0.266	N/A	N/A	C	0.265
rs1330349	116880563	Intron 6	G	0.424	G	0.416	G	0.438	G	0.448	G	0.476
rs1330351	116880743	Intron 6	A	0.488	A	0.474	A	0.477	N/A	N/A	G	0.488

SNP locations			Minor allele frequencies									
SNP	Location (bp)	Gene location	Controls		Older affected cases		Left main cases		GENECARD probands		Aorta samples	
			Minor allele	MAF	Minor allele	MAF	Minor allele	MAF	Minor allele	MAF	Minor allele	MAF
rs1757106	116885154	Intron 4	T	0.468	T	0.456	T	0.473	N/A	T	T	0.442
rs1757108	116885480	Intron 4	T	0.43	T	0.43	T	0.426	N/A	T	T	0.418
rs1757095	116888215	Exon 3 (Q539R)	T	0.076	T	0.065	T	0.053	T	0.072	T	0.046
rs2992147	116889135	Exon 3 (V232 V)	G	0.436	G	0.428	G	0.426	G	0.442	G	0.436
rs1271449	116892343	Intron 2	C	0.32	C	0.303	C	0.326	N/A	C	C	0.322
rs1272041	116895328	Intron 1	C	0.376	C	0.373	C	0.38	N/A	C	C	0.388
rs3789867	116915509	Intron 1	G	0.451	G	0.461	G	0.475	N/A	G	G	0.445
rs3748166	116919970	Intron 1	G	0.333	G	0.303	G	0.308	N/A	G	G	0.29
rs2071520	116920613	5' flanking gene	G	0.32	G	0.343	G	0.337	N/A	G	G	0.31

The rs number for each SNP is listed, followed by physical map coordinates on the NCBI human genome (build 36.3), location within the TNC locus, and the minor allele and its frequency (MAF) in each of the datasets or subsets analyzed. With SNPs that had minor allele frequencies close to 50%, the allele defined as the “minor” allele sometimes switched between datasets; these SNPs are indicated in bold. To avoid confusion with the minor allele for these and all other SNPs, we defined the minor allele in our analyses using the overall CATHGEN dataset, including both cases and controls

N/A not genotyped in GENECARD dataset

Table 3
Significant *TNC* SNP associations within the aorta samples

SNP	Raised lesions		Sudan IV staining	
	<i>p</i> value	OR (95% CI)	<i>p</i> value	OR
rs4452883	<i>0.001</i>	4.48 (1.80–11.19)	0.19	1.36
rs11787664	0.02	2.38 (1.17–4.86)	0.44	1.17
rs3789875	0.004	3.41 (1.48–7.84)	0.07	1.54
rs12347433	0.002	4.01 (1.64–9.80)	0.18	1.39
rs1547692	0.03	0.44 (0.20–0.94)	0.37	0.82
rs955450	0.007	0.29 (0.12–0.71)	0.27	0.76
rs3789873	0.01	0.34 (0.15–0.80)	0.27	0.76
rs1330349	0.01	2.62 (1.25–5.50)	0.07	1.46
rs1330351	0.02	0.41 (0.19–0.89)	0.08	0.7
rs1757106	0.05	0.47 (0.22–0.99)	0.08	0.68
rs3789867	0.02	0.37 (0.17–0.83)	0.27	0.78
rs2071520	0.01	0.34 (0.14–0.79)	0.25	0.76

The rs number for each SNP is listed, followed by the *p* value and the odds ratio (OR) in the raised lesion analysis and the *p* value in the Sudan IV analysis. The 95% confidence intervals (CI) are presented for raised lesion ORs. All *p* values that achieve statistical significance ($p < 0.05$) are indicated by bold font, and SNPs that would meet a conservative Bonferroni correction ($0.05/35 \approx 0.001$) are given in italics. Only SNPs that achieve statistical significance in the raised lesion analysis are shown. All *p* values presented represent genotypic (rather than allelic) associations

Table 4
Significant *TNC* SNP associations within the CATHGEN and GENE CARD samples

	SNP name	rs4978617	rs17239726	rs1999223	rs4452883*	rs12555312	rs3789875*	rs12347433*	rs944225
CATHGEN									
All CAD cases									
Basic model	<i>p</i> value	0.1	0.23		0.06		0.07		
	OR	1.22 (0.97–1.54)	1.19 (0.90–1.57)	0.75 (0.58–0.95)	1.26 (0.99–1.61)	0.70 (0.49–0.99)	1.25 (0.98–1.60)	1.30 (1.01–1.67)	0.77 (0.61–0.99)
Full model	<i>p</i> value	0.11	0.14	0.05	0.06	0.1	0.06	0.03	0.04
	OR	1.23 (0.96–1.58)	1.26 (0.93–1.70)	0.76 (0.59–1.00)	1.29 (0.99–1.67)	0.73 (0.50–1.07)	1.29 (0.99–1.68)	1.34 (1.02–1.75)	0.76 (0.59–0.99)
Older CAD cases									
Basic model	<i>p</i> value	0.01	0.09	0.1	0.01	0.05	0.01	0.002	0.23
	OR	1.48 (1.11–1.98)	1.35 (0.96–1.91)	0.77 (0.57–1.05)	1.46 (1.08–1.97)	0.61 (0.37–0.99)	1.46 (1.08–1.97)	1.61 (1.18–2.18)	0.83 (0.62–1.12)
Full model	<i>p</i> value	0.04	0.04	0.1	0.01	0.1	0.01	<i>0.001</i>	0.17
	OR	1.38 (1.02–1.85)	1.46 (1.02–2.11)	0.76 (0.55–1.05)	1.53 (1.12–2.09)	0.65 (0.39–1.08)	1.54 (1.13–2.10)	1.69 (1.22–2.32)	0.80 (0.58–1.10)
Left main CAD cases									
Basic model	<i>p</i> value	0.09	0.03	0.08	0.01	0.01	0.01	0.004	0.35
	OR	1.29 (0.96–1.73)	1.45 (1.03–2.03)	0.76 (0.56–1.04)	1.46 (1.09–1.97)	0.53 (0.33–0.87)	1.47 (1.09–1.99)	1.56 (1.15–2.11)	0.87 (0.65–1.17)
Full model	<i>p</i> value	0.21	0.01	0.08	0.01	0.03	0.01	0.005	0.24
	OR	1.22 (0.90–1.67)	1.58 (1.10–2.26)	0.74 (0.53–1.04)	1.48 (1.08–2.03)	0.55 (0.32–0.93)	1.51 (1.10–2.08)	1.59 (1.16–2.20)	0.83 (0.60–1.13)
GENE CARD									
Probands									
Basic model	<i>p</i> value	0.51	0.2	N/A	N/A	0.03	0.04	0.02	0.17
	OR	1.08 (0.86–1.35)	1.20 (0.91–1.58)	N/A	N/A	0.66 (0.46–0.95)	1.29 (1.01–1.64)	1.33 (1.04–1.70)	0.85 (0.67–1.07)
Full model	<i>p</i> value	0.18	0.25	N/A	N/A	0.06	0.09	0.07	0.13
	OR	1.19 (0.92–1.52)	1.20 (0.88–1.63)	N/A	N/A	0.68 (0.46–1.02)	1.27 (0.97–1.67)	1.29 (0.98–1.70)	0.82 (0.63–1.06)
Families									
APL	<i>p</i> value	0.67	0.88	N/A	N/A	0.92	0.05	0.08	0.28

The rs number for each SNP is listed, followed by the *p* value and the odds ratio (OR) within each analysis. 95% confidence intervals (CI) are presented for each OR in parentheses. All *p* values that achieve statistical significance ($p < 0.05$) are indicated by bold font, and SNPs that would meet a conservative Bonferroni correction ($0.05/35 \approx 0.001$) are given in italics. Only SNPs that achieved statistical significance in at least one dataset or sub-analysis of a dataset are shown. Results from both the basic (adjusted for sex) and full (adjusted for sex and known cardiovascular risk factors) models are presented. All *p* values presented represent genotypic (rather than allelic) associations

N/A SNP not genotyped in the GENE CARD probands

* SNPs that achieved statistical significance in the aorta raised lesion analysis