

Brief report

The thrombospondin-1 N700S polymorphism is associated with early myocardial infarction without altering von Willebrand factor multimer size

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The N700S polymorphism of thrombospondin-1 (TSP-1) has been identified as a potential genetic risk factor for myocardial infarction (MI). In a large case-control study of 1425 individuals who survived a myocardial infarction prior to age 45, the N700S polymorphism was a significant risk factor for myocardial infarction in both homozygous (odds ratio [OR] 1.9, 95% confidence interval [CI] 1.1-3.3, $P = .01$) and heterozy-

gous carriers of the S700 allele (OR 1.4, 95% CI 1.1-3.3, $P = .01$). TSP-1 has been shown to reduce von Willebrand factor (VWF) multimer size, and the domain responsible for VWF-reducing activity has been localized to the calcium-binding C-terminal sequence. As the N700S polymorphism was previously shown to alter the function of this domain, we investigated whether the altered VWF-reducing activity of TSP-1 underlies

the observed prothrombotic phenotype. The TSP1 N700S polymorphism did not influence VWF multimer size in patients homozygous for either allele nor was there a significant reduction of VWF multimer size following incubation with recombinant N700S fragments or platelet-derived TSP-1. (Blood. 2006;108:1280-1283)

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Introduction

The genetic defect(s) underlying a hereditary predisposition for myocardial infarction (MI) has been frustratingly difficult to identify and corroborate. Multiple studies have demonstrated an association of a specific polymorphism in a candidate gene, only to be refuted when analyzed in a different cohort. One such candidate polymorphism was identified by Topol et al¹ in a survey of 72 single-nucleotide polymorphisms (SNPs) from 62 candidate genes in 398 sibling pairs. Homozygosity for a missense variant in thrombospondin-1 at amino acid 700, resulting in an asparagine-to-serine substitution (N700S), was associated with an adjusted odds ratio for myocardial infarction of 8.66 in their cohort. However, this association was not confirmed in subsequent studies, which may be a reflection of the low frequency of the S700 allele and insufficient power to detect a significant association.²⁻⁴ Therefore, we sought to evaluate the risk of MI associated with the TSP-1 N700S variant in a large cohort of individuals who survived an MI prior to age 45.

Thrombospondin-1 (TSP-1) is a glycoprotein stored in human platelet alpha granules. The precise role TSP-1 plays in platelet aggregation is unclear but may involve stabilization of fibrinogen-platelet cross-bridges.⁵ Thrombospondin-1 also has been shown to regulate von Willebrand factor (VWF) multimer size.^{6,7} Larger VWF multimers are hemostatically active and bind to collagen and platelets with approximately 100-fold greater affinity than monomeric VWF.⁸ This is exemplified by the microvascular thrombosis seen in patients with thrombotic thrombocytopenic purpura (TTP)

following the accumulation of ultralarge VWF multimers. Pimanda et al⁷ identified the TSP-1 domain responsible for VWF-reducing activity in the calcium-binding C-terminal sequence at position Cys 974. Considering the proximity of the N700S TSP1 polymorphism to this domain and the demonstration that S700 alters the calcium-binding affinity of this domain,⁹⁻¹¹ we also investigated whether altered VWF-reducing activity of the TSP-1 N700S variant contributes to the observed prothrombotic phenotype.

Study design

Case-control study

As previously described, this case-control study involved 125 Italian coronary care units and enrolled 1425 individuals who survived a first episode of MI prior to age 45.¹² The 1425 healthy controls were matched for age, sex, geographic origin, and freedom from ischemic heart disease based on history, physical examination, and stress testing.

Genotyping of TSP-1 for N700S polymorphism

Genomic DNA was isolated by standard methods. Detection of the N700S SNP was performed using site-specific TaqMan-labeled probes and analyzed on an ABI Prism 9700 sequence detection system (Applied Biosystems, Foster City, CA).

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Table 1. N700S genotype analysis in young survivors of MI

Genotype	Patients no. (%)	Controls no. (%)	OR (95% CI)
AA	1158 (81.3)	1217 (85.4)	—
AG	250 (17.5)	199 (14.0)	1.4 (1.1-1.8)*
GG	17 (1.2)	9 (0.6)	1.9 (1.1-3.3)*
Allele frequency G	0.10	0.08	1.3 (1.1-1.6)†

For patients and controls, N = 1425 each. Unadjusted OR for homozygote (GG) and heterozygote (AG) genotype as compared to the AA genotype estimated under a multiplicative model. Adjusted OR for G allele: 1.4 (95% CI 1.1-1.8; $P < .01$).

* $P = .01$.

† $P < .01$.

TSP-1 and recombinant proteins

Human TSP-1 was purified from the supernatant of thrombin-treated platelets.¹³ Recombinant VWF containing high molecular weight multimers was a generous gift of Dr Peter Turecek (Baxter Bioscience, Vienna, Austria). Recombinant TSP-1 fragments were prepared by polymerase chain reaction (PCR) using full-length cDNA from human TSP-1. The TSP-1 fragments included residues 648-1170. The N700S polymorphism was generated by PCR mutagenesis as described by Hannah et al¹⁰ and expressed in *Drosophila* S2 cells by methods described elsewhere.¹⁴ Conditioned media was applied to ProBond nickel-chelating resin (Invitrogen, Carlsbad, CA) and eluted with imidazole 500 mM in HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (20 mM HEPES, NaCl 500 mM, CaCl₂ 2 mM, pH 7.4) followed by dialysis against HEPES buffer. Recombinant ADAMTS13 was generated using human embryonic kidney cells.¹⁵

VWF multimer analysis

Aliquots of patient plasma samples were diluted in HEPES buffer (50 mM HEPES, 125 mM NaCl, 0.1 mM, or 2 mM CaCl₂) and assayed for collagen-binding activity (CBA) and VWF antigen based on methods described by Favalaro et al.¹⁶ VWF multimeric pattern was analyzed according to conditions described by De Cristofaro et al¹⁵ and the percentage of high molecular weight multimers calculated by densitometry.¹⁷

Free thiol detection

The molar absorptivity of the N700S fragments (~10 μM) was measured at 412 nm using 4 mg/mL DTNB (55'-dithiobis-[2-nitrobenzoic acid]) reagent (Pierce, Rockford, IL) in HEPES buffer with or without 10 mM EDTA (ethylenediaminetetraacetic acid).⁷

Statistical considerations

The association between the S700 allele and acute MI was estimated by calculating an odds ratio and adjusted for traditional, nongenetic factors as previously described.¹² The study was sufficiently powered (80%) to detect a true difference between populations assuming an OR more than 1.38 and allele frequency of at least 10% using an uncorrected χ^2 test. Comparisons of parametric data were performed using *t* test for independent samples. The in vitro reduction of VWF multimers by TSP-1 proteins was assessed by CBA and significance calculated by ANOVA 1-way analysis with multiple comparisons against the control group (VWF alone).

Results and discussion

Genetic association studies for complex trait diseases have been plagued by lack of reproducibility. The basis for these discordant findings is multifactorial, but one common criticism is insufficient sample size.¹⁸⁻²¹ In this report, we confirm an association between MI and the TSP1 N700S polymorphism in more than 1400 young survivors of MI (Table 1). The S700 allele was shown to be a significant risk factor for MI in homozygous individuals (OR 1.9, 95% CI 1.1-3.3) and, for the first

time, heterozygosity also was observed to be a significant risk factor for MI (OR 1.4, 95% CI 1.1-1.8).

Several groups now have shown that the TSP1 N700S polymorphism results in a conformational change that affects the calcium-binding properties of the glycoprotein.⁹⁻¹¹ These findings are in line with the observation that a homologous asparagine residue in thrombospondin-2 coordinates a calcium ion.²² Similar missense mutations in the calcium-binding domain of thrombospondin-5 affect its conformation and result in its retention in the endoplasmic reticulum.²³ In differentiated chondrocytes, but not in other cells that generate thrombospondin-5, the accumulation of mutant protein leads to dysfunction of the endoplasmic reticulum and cell death, which, in turn, leads to skeletal dysplasia. Our observation that the TSP1 S700 allele was associated with coronary disease in both homozygous and heterozygous carriers parallels the dominant negative pattern of phenotypic expression of these TSP-5 mutations.²⁴ The molecular underpinnings of the TSP1 N700S polymorphism and coronary artery disease continue to be investigated. Narizhneva et al²⁵ observed that the S700 variant led to an increase in the binding affinity of TSP-1 for fibrinogen corresponding with greater TSP-1 surface expression on platelets. We assessed whether

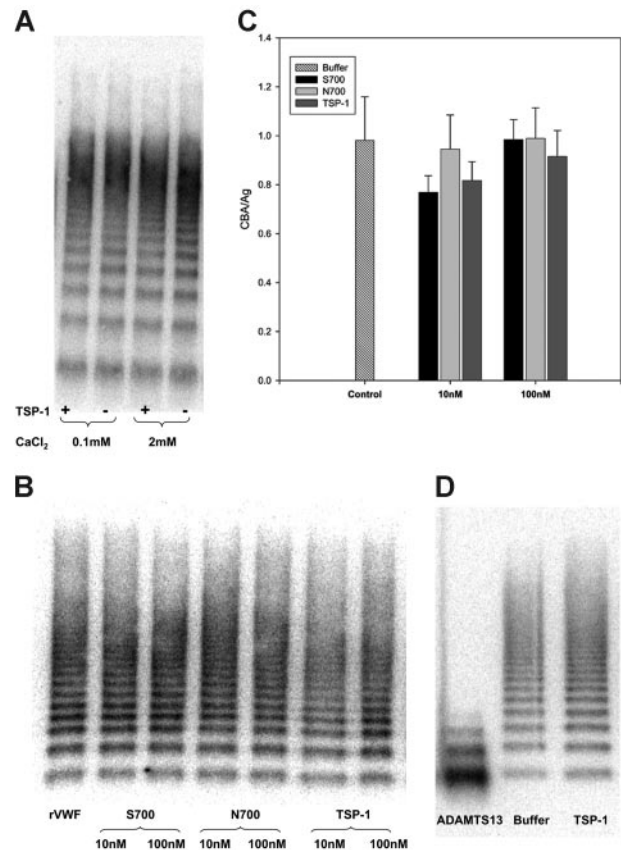


Figure 1. VWF multimer analysis following incubation with TSP-1 proteins. (A) 100 nM of TSP-1 in HEPES buffer (50 mM HEPES, 0.125 M NaCl, and 0.1 mM or 2 mM CaCl₂) was incubated with TTP plasma for 1 hour at 37°C and then directly visualized by gel electrophoresis. The percentage of high molecular weight multimers was not affected by the addition of TSP-1 ($P = .50$) as measured by densitometry. (B-C) Recombinant VWF (0.4 U/mL) was incubated with full-length TSP-1 or N700S recombinant protein (10 nM or 100 nM) for 1 hour at 37°C. Controls were incubated with HEPES buffer alone. No significant reduction was observed in VWF multimer size by gel electrophoresis or as measured by collagen-binding activity relative to VWF antigenic concentrations (ANOVA 1-way analysis, $P = .08$). Error bars represent standard deviations. (D) rVWF (0.4 U/mL) was incubated overnight with recombinant ADAMTS13 (10 μg/mL) or platelet TSP-1 (100 nM) at 37°C in Tris (tris[hydroxymethyl]aminomethane) buffer (10 mM Tris, 1.5 mM urea, 3 mM BaCl₂, 0.1 mM CaCl₂).

Table 2. N700S VWF multimer analysis in young survivors of MI

	MI patients		Controls		P*
	S700	N700	S700	N700	
No. of patients	17	9	9	9	NA
HMW % (95% CI)	31.2 (29.1-33.3)	28.7 (25.0-32.4)	31.1 (27.1-35.0)	29.9 (25.8-33.9)	.20
CBA/Ag (95% CI)	1.0 (0.9-1.0)	1.0 (0.9-1.2)	1.0 (0.8-1.1)	1.0 (0.9-1.2)	.20

HMW indicates percentage of high-molecular-weight VWF multimers measured by densitometry; CBA/Ag, collagen-binding activity relative to VWF antigen concentration; NA, not applicable.

*S700 versus N700.

the *TSP1 N700S* polymorphism contributed to a prothrombotic phenotype by influencing TSP-1–mediated regulation of VWF multimer size.

Full-length platelet-purified TSP-1 was incubated with TTP plasma, but there was no demonstrable reduction in plasma VWF multimer size by gel electrophoresis (Figure 1A) or CBA (data not shown). Increasing the calcium concentration did not alter these findings. Because plasma contains endogenous TSP-1, which may confound the results, assays then were conducted using recombinant VWF with an intact multimeric structure (rVWF). As shown in Figure 1B and C, the incubation of TSP-1 S700 or N700 fragments and platelet-purified full-length TSP-1 with rVWF did not significantly reduce multimer size as measured by gel electrophoresis or CBA ($P = .08$). This contrasts with the ADAMTS13 (a disintegrin-like and metalloprotease with thrombospondin type 1-motif 13)–mediated digestion of rVWF as illustrated in Figure 1D.

To evaluate whether the presence of the N700S TSP-1 variant influenced VWF multimer size in vivo, multimer analysis was performed using plasma samples from individuals homozygous for either allele. As shown in Table 2, the relative percentage of high molecular weight VWF multimers did not differ between individuals homozygous for either S700 or N700 alleles ($P = .20$) nor among those individuals who suffered an MI ($P = .19$). These findings were confirmed using a functional assay to assess collagen-binding activity of VWF relative to VWF antigenic concentrations ($P = .20$).

Our inability to visualize a reduction in VWF multimer size following the addition of either full-length platelet-purified or recombinant TSP-1 proteins differs from 2 previous reports.^{6,7} The only apparent methodologic difference was that the purification of the peptides was performed at higher CaCl_2 concentrations (2 mM), because we observed significant protein degradation at 0.1 mM CaCl_2 , presumably due to the calcium-dependent conformational changes.²⁶ We also chose to investigate the differential activity of TSP-1 in modulating VWF multimer size in conditions similar to plasma, where the normal calcium concentration is slightly higher than 2 mM.²⁷ Notably, the crystal structure of the C-terminal

domain of TSP-1 was recently elucidated, and in the calcium-replete state, the thiol group at position 974 appears to be buried below a $\beta 1$ - $\beta 2$ loop, obstructing its availability for disulfide bond reduction.²⁸ The number of free thiols in the TSP-1 N700S fragments was quantified using DTNB reagent; in the absence of calcium (10 mM EDTA), there was approximately 1 mole of thiol per mole N700S fragment, consistent with published reports.⁷ In the presence of 2 mM CaCl_2 , the number of free thiols detected was reduced by 42% for both N700 and S700 fragments.

The initial report of TSP-1 reduction of VWF was made using TTP plasma as the source of ultralarge VWF.⁶ Platelet activation, a central feature of TTP, results in a hundred-fold increase in serum TSP-1 levels.²⁹ Therefore, the TSP-1–mediated reduction of VWF in a TTP plasma sample in vitro is of uncertain physiologic significance, since the presence of ultralarge VWF is a characteristic finding of TTP. Pimanda et al³⁰ also observed that TSP-1^{null} mice paradoxically have smaller, rather than larger, circulating VWF multimers, a phenomenon that has been ascribed to a TSP-1 and ADAMTS13 interaction. Although the influence of the TSP-1 N700S polymorphism on ADAMTS13 activity was not directly assessed in this study, we did not observe a difference in the size of VWF multimers in plasma obtained from patients homozygous for the S700 allele; this argues against a significant effect via a TSP-1 and ADAMTS13 interaction under physiologic conditions. In summary, we found that the S700 allele was a risk factor for coronary thrombosis in a large case-control study of young survivors of MI but were unable to demonstrate an effect on VWF multimer size.

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