Polymorphism in the protease-activated receptor-4 gene region associates with platelet activation and perioperative myocardial injury

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Protease-activated receptors (PAR)-1 and -4 are the principal receptors for thrombin-mediated platelet activation. Functional genetic variation has been described in the human PAR1 gene, but not in the PAR4 gene (*F2RL3*). We sought to identify variants in and around *F2RL3* and to determine their association with perioperative myocardial injury (PMI) after coronary artery bypass graft surgery. We further explored possible mechanisms for *F2RL3* single nucleotide polymorphism (SNP) associations with PMI including altered receptor expression and platelet activation. Twenty-three SNPs in the *F2RL3* gene region were genotyped in two phases in 934 Caucasian subjects. Platelets from 43 subjects (23 major allele, 20 risk allele) homozygous for rs773857 (SNP with the strongest association with PMI) underwent flow cytometry to assess PAR4 receptor number and response to activation by a specific PAR4 activating peptide (AYPGKF) measured by von Willebrand factor (vWf) binding and P-selectin release and PAC-1 binding. We identified a novel association of SNP rs773857 with PMI (OR = 2.4, *P* = 0.004). rs773857 risk allele homozygotes have significantly increased platelet counts and platelets showed a significant increase in P-selectin release after activation (*P* = 0.004). We conclude that rs773857 risk allele homozygotes are associated with risk for increased platelet count and hyperactivity. Am. J. Hematol. 00:000–000, 2011. © **2011 Wiley Periodicals, Inc.**

Introduction

An estimated one million people worldwide annually experience nonfatal perioperative myocardial injury (PMI) or death [1]. PMI is associated with increased postoperative length of intensive care unit and hospital stays, short and long-term morbidity and mortality, as well as increased healthcare resource utilization [2–6].

After coronary artery plaque rupture, activated platelets play a crucial role in the propagation of thrombosis, and therefore the exacerbation of PMI. The exposure of coronary vascular subendothelium and plaque contents during coronary arterial plaque rupture activates the hemostatic system and locally generates thrombin. In response, circulating platelets roll on exposed subendothelium, adhere and form aggregates. These processes involve platelet receptors such as von Willebrand factor receptor complex, granule secretion, and integrins such as αIIbβ3 [7].

Thrombin, an extracellular protease, activates platelets by irreversible cleavage of the N-terminus of the class of protease-activated receptors (PAR) -1, -3, and -4. PARs belong to a G protein-coupled receptor family and serve as cellular targets of thrombin signaling to platelets and endothelium, via cell surface expression. PAR1 and PAR4 are expressed on human platelets and are the principal receptors for thrombin-mediated platelet activation. Functional genetic variation has been described in the human PAR1 gene (*F2R*), but not in the PAR4 gene (*F2RL3*) [8–10].

Functional variation in the human platelet genome affects cell adhesion, cell activation, and cell-to-cell contact interactions and has been implicated in cardiovascular disease, arterial thrombosis, and myocardial infarction [11–17]. Furthermore, platelet reactivity is a significant predictor of cardiac outcomes after myocardial infarction [18,19]. In coronary artery bypass grafting (CABG) and vascular surgery patient populations, genetic variation of platelet membrane receptor (glyco) proteins has been associated with PMI [17,20–22].

Our aim was to determine the relationship between platelet genetic variation in the thrombin receptor *F2RL3* gene and incidence of PMI in subjects undergoing primary CABG surgery. We hypothesized that *F2RL3* variants may be associated with PMI through a causal mechanism of altered receptor expression, platelet activation, and thrombin signaling.

Methods

Study population. Two institutions (Brigham and Women's Hospital and the Texas Heart Institute) recruited subjects aged 20–90 years undergoing non-emergent primary CABG surgery with cardiopulmonary bypass (CPB), without other concurrent surgery (http://clinicaltrials.gov/show/NCT00281164) between August 2001 and September 2006. Subjects with a preoperative hematocrit <25% or who received transfusion of leukocyte-rich blood products within 30 days before surgery were not enrolled. To avoid potential influence of population stratification on observed associations, analysis was restricted to subjects who self-

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Additional Supporting Information may be found in the online version of this article.

Conflict of interest: Nothing to report.

S.C.B. and K.M.H. are joint seniors.

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TABLE I. Patient Characteristics, Stratified by Perioperative Myocardial Injury

Preoperative characteristic	No PMI ($N = 616$)	PMI ($N = 69$)	P value
Age (years)	65 ± 10	65 ± 10	
Male gender	82% (505)	80% (55)	0.62
Institution			
Institution 1	90% (505)	10% (57)	
Institution 2	90% (111)	10% (12)	1
Diabetes			
Insulin dependent	8% (52)	9% (6)	
Non-insulin dependent	21% (132)	12% (8)	0.15
Hypertension	76% (465)	75% (52)	1
Hypercholesterolemia	76% (470)	74% (50)	0.65
BMI (kg/ m^2)	29.2 ± 5.4	29.3 ± 5.5	0.97
Preoperative creatinine (mg/dL)	1.09 ± 0.3	1.12 ± 0.4	0.92
Myocardial infarction $\leq$ 2 weeks preoperatively	16% (98)	36% (25)	$<$0.001
LV ejection fraction (%)	52 ± 13	47 ±12	0.002
Coronary artery regions with $>$ 50% stenosis			
1–2 regions	29% (181)	25% (17)	
3 regions	52% (323)	54% (37)	
$\geq$4 regions	18% (112)	22% (15)	0.63
Preoperative medications			
Aspirin	75% (460)	74% (51)	0.88
Non-aspirin platelet inhibitor	17% (103)	25% (17)	0.13
Statin preop	77% (472)	71% (49)	0.30
Intravenous heparin	25% (152)	39% (27)	0.01
Intravenous nitrate	12% (73)	22% (15)	0.03
Warfarin $<$ 7 days preoperatively	5% (31)	3% (2)	0.76
Preoperative laboratory data			
Hematocrit (%)	39.9 ± 4.6	39.7 ± 4.9	0.77
Platelet count (10^6/mL)	238 ± 70	234 ± 69	0.47
cTnI (μg/L)	0.16 ± 0.66	3.8 ± 10.5	$<$0.001
Intraoperative characteristics			
Cardiopulmonary bypass time (minutes)	95 ± 36	116 ± 44	$<$0.001
Aortic cross clamp time (minutes)	71 ± 29	86 ± 36	0.002
Lowest body temperature (°C)	33 ± 2	32 ± 3	0.43
Number of coronary grafts			
1–2	15% (19)	12% (8)	
3	47% (291)	52% (36)	
$\geq$4	38% (234)	36% (25)	0.67
Postoperative laboratory data			
Hematocrit POD1(%)	29.5 ± 3.8	29.8 ± 3.6	0.42
Platelet count POD3 (10^6/mL)	171 ± 60	153 ± 54	0.02
cTnI postop Day 1 (μg/L)	1.87 ± 1.8	24.1 ± 16.3	$<$0.001

PMI defined as cardiac Troponin I (cTnI) $>$ 90% percentile on postoperative day 1 after primary CABG surgery.

POD, post-operative day; LV, left ventricle; BMI, body mass index.

reported four Caucasian grand-parental ancestry. Study protocols were approved by respective Institutional Review Boards, and participants were enrolled following informed written consent.

Demographic and clinical data collection. At each site, patient demographics, perioperative risk factors, medications, and postoperative outcomes were recorded using study-specific case report forms. Blood samples were drawn before induction of general anesthesia and on the morning of postoperative day (POD) 1. Serum and plasma were stored in vapor phase liquid nitrogen until analysis. Cardiac troponin I (cTnI) was measured at a single blinded core facility using the sandwich immunoassay Triage® platform (Biosite, San Diego, CA).

Genetic association study. F2RL3 genotyping. DNA was extracted from white blood cells using standard procedures. Genotyping was performed in two phases using the iPLEX Gold assay on a MassARRAY system (Sequenom, San Diego, CA) in accordance with the manufacturer's standard recommendations. Automated genotype calling was done with MassARRAY Typer 4. Genotype clustering was visually checked by an experienced evaluator. Single nucleotide polymorphism (SNPs) with a genotyping call rate $<$95%, with significant deviation from Hardy–Weinberg equilibrium ($P < 0.001$ in controls) and nonrandom missingness ($P < 0.05$) between cases and controls, were excluded from subsequent analysis. After exclusions and quality control, Phase 1 included 685 subjects and 23 SNPs (Supporting Information Table I). The 23 candidate SNPs were selected utilizing publicly available information from NCBI (http://www.ncbi.nlm.nih.gov/), the HapMap [23], SeattleSNPs [24], and SNPper [25] to obtain comprehensive coverage of the *F2RL3* gene and its flanking regions. SNPs representative of one haplotype block upstream and downstream of *F2RL3* were chosen. Linkage disequilibrium (LD) tagging SNPs with minor allele frequencies of 5% or greater in the HapMap Caucasian cohort were identified using Tagger [26]. Preference was given to the following criteria: (i) non-synonymous coding variation, (ii) promoter region variation, (iii) variation in the 3' untranslated region, (iv) variation at splice junctions, (v) haplotype tagging SNPs, and (vi) previously identified candidate SNPs. The *F2RL3* gene does not have identified copy number variations or candidate microsatellite polymorphisms.

Phase 2 included genotyping of 10 of the original 23 SNPs in 934 subjects, which included all subjects from Phase 1 and an additional 249 subjects (Supporting Information Table I). The 10 SNPs genotyped in Phase 2 were selected from the 23 Phase 1 SNPs using Tagger [26], based on their association with PMI and their LD to the SNPs from Phase 1 with the goal of including the most significant SNPs. All SNPs with a P-value $<$0.05 in univariate analysis and an LD $r^2 > 0.8$ were included.

Functional platelet analysis. Functional platelet analyses were performed using whole blood drawn from 43 male subjects selected from the Phase 1 cohort. These subjects were homozygous for the major or minor alleles of SNP rs773857 (23 major allele, 20 risk allele), the *F2RL3* locus SNP with the most significant association with PMI in both Phase 1 and Phase 2 assessments. To ensure platelet viability, recruitment was limited to those subjects living within 1.5 h of the single laboratory where the functional analysis was performed as blood draws were performed at subjects' homes for their convenience. Subjects were divided into homozygous major allele and homozygous risk allele and matched by age (±10 years) and by their POD 1 cTnI level (above or below median) after their CABG surgery. Analysis was limited to male subjects to avoid confounding by gender in a population of 82% male subjects.

PRP (platelet rich plasma) preparation. All phlebotomy for the functional analyses was performed between 7:00 am and 10:00 am with a 21 g needle using aseptic technique. Nine milliliters of whole blood was collected in two buffered 0.105 M sodium citrate blood collection tubes (BD Vacutainer, Franklin Lakes, NJ) within 2 h of first centrifugation. Human PRP was obtained by centrifugation of the blood at 100*g* for 20 min at room temperature. PRP preparation and all further analyses were performed blinded to patient genotype.

Flow cytometry. Platelet count was determined by flow cytometry using reference beads (SPHERO rainbow fluorescent beads, 5.5-μm diameter, Spherotech, Libertyville, IL) [27]. PAR1 and PAR4 receptors were detected on resting and activated platelets in PRP using monoclonal (IgG) anti human PAR1 (Santa Cruz Biotechnology, Santa Cruz, CA) and PAR4 (Abnova, Taipei City, Taiwan) antibodies. Platelet surface receptor numbers were quantified using a Calibrator kit (Platelet Calibrator, Biocytex, Marseille, France). To quantify receptor expression, a calibration curve was constructed for each PRP sample analysis series using the specific PAR1 and 4 antibodies and the appropriate included negative isotype control. Antibody labeling of platelets was performed for 20 min at room temperature with 20 μg/μL of anti-human mAbs against PAR1 and PAR4 and appropriate isotype controls. Platelet samples were analyzed on a FACSCalibur flow cytometer (BD, Franklin Lakes, NJ). Five thousand events were acquired and data were analyzed using CellQuest® software (BD). Receptor numbers were derived from the calibration curve obtained, after subtracting the negative isotype control value [9].

Platelet activation. Freshly obtained PRP was activated via PAR1 or PAR4 receptors using 3 μg/mL of thrombin receptor activating peptide (TRAP) specific for PAR1 (SFLLRN, obtained from Bachem, Bubendorf, Switzerland) or 18 μg/mL TRAP specific for PAR4 (AYPGKF, obtained from Bachem) for 3 min at 37°C. After stimulation, platelets were stained with 5 μg/mL of FITC-labeled anti human P-Selectin (CD62P, Becton Dickinson) or PE-labeled anti human αIIbβ3 (PAC-1, BD) mAbs, or with 5 μg/mL of FITC-labeled anti human von Willebrand factor (vWf) polyclonal antibody (Dako, Carpinteria, CA) for 20 min at room temperature and directly analyzed on a FACSCalibur (BD), as described above. The appropriate isotype for each antibody were used as control.

Statistical analysis for SNP association. Potential population stratification between Northern versus Southern European origins was examined using SNPs within the lactase gene (*LCT*—rs182549, rs2322659, rs3754689, rs3769005, rs4954490, rs4988235) known to vary in frequency along a European north–south cline. The results did not show significant heterogeneity; therefore, a pooled analysis across Northern and Southern European groups was performed.

Categorical and continuous demographic characteristics were compared between groups with likelihood ratio χ^2 and Wilcoxon rank sum

TABLE II. Patient Characteristics of 43 Subjects Selected for Functional Platelet Analysis

	Major allele (n = 23)	Risk allele (n = 20)	P value
Preoperative characteristic			
Age at enrollment	66 ± 11	61 ± 9	0.08
Age at blood draw	72 ± 11	67 ± 9	0.08
Hypercholesterolemia	87% (20)	80% (16)	0.69
Diabetes			
Insulin dependent	4% (1)	0% (0)	
Non-insulin dependent	9% (2)	35% (7)	0.06
Hypertension	78% (18)	65% (13)	0.5
BMI (kg/m^2)	28.0 ± 2.5	28.2 ± 2.5	0.83
Smoker past	87% (20)	90% (18)	1
Previous myocardial infarction	35% (8)	25% (5)	0.53
Myocardial infarction $\leq$ 2 weeks preoperatively	9% (2)	15% (3)	0.65
Preoperative LVEF %	54 ± 10	53 ± 11	0.85
Coronary artery regions with $>$ 50% stenosis			
1–2 regions	26% (6)	40% (8)	
3 regions	48% (11)	55% (11)	
$\geq$4 regions	26% (6)	5% (1)	0.16
Preoperative laboratory data			
Blood Type (O^- vs. other)	48% (11)	55% (11)	0.76
Hematocrit (%)	39.7 ± 4.5	40.0 ± 3.6	0.81
Creatinine (mg/dL)	1.1 ± 0.1	1.1 ± 0.2	0.97
Platelet count (10^6/mL)	225 ± 39	250 ± 45	0.07
cTnI (μg/L)	0.1 ± 0.2	0.3 ± 0.6	0.84
Platelet count POD3 (10^6/mL)	172 ± 41	189 ± 45	0.43
cTnI POD1 (μg/L)	2.5 ± 4.8	1.8 ± 1.1	0.71
Intraoperative characteristics			
Cardiopulmonary bypass time (min)	98 ± 24	106 ± 22	0.22
Aortic cross clamp time (min)	74 ± 16	83 ± 20	0.11
Number of coronary grafts			
1–2	4% (1)	10% (2)	
3	48% (11)	45% (9)	
$\geq$4	48% (11)	45% (9)	0.72

BMI, body mass index; POD, post-operative day; LVEF, left ventricular ejection fraction; cTnI, cardiac troponin I.

tests, respectively. A multivariable logistic regression analysis was used to test the association between PAR4 variants and PMI, defined as POD 1 cTnI in the top 10th percentile of the examined Phase 1 study cohort (cTnI $>$ 6.73 mg/L), a definition we have utilized before [6]. Clinical and demographic covariates associated with PMI after cardiac surgery in prior studies were included in the multivariate model with stepwise selection (age, gender, institution, preoperative statin, acetylsalicylic acid, or platelet inhibitor use, recent MI, preoperative creatinine, CPB time, and number of grafts). Permutation-based empirical P-values were used to adjust for SNP data distribution (point-wise) and multiple SNP association tests (family-wise).

PLINK (version 1.04) [28], SAS and SAS/Genetics (SAS Institute, Cary, NC) were used for genetic analyses. Hardy-Weinberg equilibrium was evaluated using an exact test. After application of genotype quality control criteria, univariate analyses were carried out for each SNP to test the null hypothesis of no association between marker polymorphism and PMI, based on additive, dominant and recessive genetic models. Tests of frequency association were estimated with chi-square statistic.

Statistical analysis for platelet activation. Gated and mean fluorescence intensity (MFI) quantitative flow cytometry data of platelet activation with PAR1 and PAR4 agonists was analyzed with linear regression with and without adjusting for age at the time of blood draw. In addition to the raw flow cytometry measurements, the control at time 0 and the baseline measurement subtracted from the raw measurements were also analyzed. To account for the variability of platelet counts among subjects, the flow cytometry measurements were normalized by the platelet counts from each individual.

To avoid potential false positive results driven by distribution outliers or departure from normality, the same analysis on the rank-transformed [29] and quantile-normalized [30] flow cytometry data were also applied. A two-sided $P < 0.05$ was considered significant. Statistical analyses were performed using SAS version 9.1.3 (SAS Institute, Cary, NC).

Power analysis. Since no data on PAR4 receptor density exists in humans, prior published data on PAR1 receptor polymorphisms was used as the basis for the power analysis. Prior work comparing receptor numbers for the *F2R* intronic polymorphism (IVSn-14 A/T) had shown a mean PAR1 density on human platelets of 1297 ± 186 receptors/platelet for major allele homozygotes and 857 and 1022 for the two minor allele homozygotic individuals [9,10]. A 20% variation in the number of PAR1 receptors for this polymorphism was assumed, which would also be observed in relation to our identified PAR4 variants. Therefore, the sample size to detect a difference of 150 receptors/platelet was estimated, with a standard deviation of 200 receptors/platelet, a Type I error of 0.05 and a power of 80%. Within these assumptions, the required sample would be 42 subjects; 21 subjects for each of the two groups (homozygote major and minor alleles).

TABLE III. Functional Analysis: Release of Markers Upon PAR4 Agonist Stimulation

	PAR4 (*F2RL3* rs773857)			
	Univariate		Adjust for age	
	Beta	P-value	Beta	P-value
P-Selectin				
Transformed	42.96	0.001	37.77	0.004
Non-transformed	39.72	0.006	35.70	0.014
PAC-1				
Transformed	−1.54	0.935	−5.65	0.771
Non-transformed	−18.45	0.358	−18.95	0.364
vWF				
Transformed	−33.72	0.200	−42.18	0.119
Non-transformed	−29.44	0.297	−35.16	0.230

Shown is the beta for mean fluorescence intensity. All analyses are adjusted for platelet number.

To avoid potential false positive results driven by distribution outliers or departure from normality, the flow cytometry data was rank transformed [29] and quantile-normalized [30].

Results

Of the 1,100 eligible Caucasian subjects enrolled into the source cohort during the study period, 415 were excluded from analysis in Phase 1 for one or more of the following exclusion criteria: no CABG or no CPB (n = 40), no aortic cross-clamp (n = 80), emergency surgery (n = 25), prior cardiac surgery (n = 13), CPB not used (n = 32), unplanned concurrent valve surgery performed (n = 130), missing genotype (n = 124), genotyping call rate $<$95% (n = 38), leaving 685 subjects for Phase 1 analysis. Perioperative patient characteristics of the 685 subjects included in the study analysis are shown in Table I and are stratified by occurrence of PMI. For Phase 2, an additional 249 Caucasian subjects undergoing non-emergent, isolated primary CABG with CPB were genotyped, increasing the total number of subjects in Phase 2 analysis to 934.

Clinical predictors of perioperative myocardial injury

In the Phase 1 SNP association study, subjects with PMI (n = 69) were more likely to have had a recent myocardial infarction, higher preoperative cTnI, lower preoperative left ventricular ejection fraction, have received preoperative intravenous heparin or nitrates, longer CPB and aortic cross clamp times, and lower postoperative platelet counts on (Table I). After adjusting for other covariates in multivariable analysis, only recent myocardial infarction (within 2 weeks) and length of CPB were independently associated with PMI, accounting for 9% of the risk of PMI (Supporting Information Table II).

Genomic predictors of perioperative myocardial injury

In Phase 1, only rs773857 had a significant association with PMI (point-wise empirical $P < 0.01$), and approached significance after correcting for multiple SNP association tests (family-wise empirical P = 0.057) (Supporting Information Table III). In Phase 2 analysis, rs773857 remained highly significant after multiple comparison correction [OR = 2.4; IQR = (1.4–2.0); family-wise empirical P = 0.004].

F2RL3 rs773857 risk allele associated with increase in platelet number and increased platelet α-degranulation

No significant differences in demographic or clinical variables were identified between subjects homozygous for the major allele or risk allele of rs773857 (Table II). Subjects homozygous for the risk allele rs773857 had a higher median platelet count than the homozygous major allele group (3.6×10^7 /mL vs. 3.1×10^7 /mL, $P = 0.024$) (Fig. 1). No differences in platelet size or density (not shown) or in PAR1 and PAR4 receptor platelet surface expression were detected, as determined by flow cytometry between major allele or risk allele (Fig. 1). Next, the function of major allele and risk allele platelets was investigated. P-selectin platelet surface expression was measured by flow cytometry to evaluate α-alpha-granule secretion. Although PAR1 and PAR4 receptor expression was normal in subjects with the risk allele, upon activation with the specific PAR4 agonist (AYPGKF) platelets obtained from patients with the *F2RL3* rs773857 risk allele (PAR4) had more measurable surface P-Selectin, indicating that platelets obtained from the risk group released more α-granule content compared with major allele controls. These data were consistent in both non- and rank-transformed and non- and quantile-normalized univariate analysis ($P = 0.001$) and after adjusting for age and platelet number ($P = 0.004$) (Table III). No significant difference in αIIbβ3 activation or vWf binding was identified by flow cytometry using PAC-1 or anti-vWf antibodies (Table III), although an increase in vWf binding was observed in the risk. No differences were observed between the groups when platelets were activated via the collagen receptor GPVI using convulxin. Ninety-five percent of subjects were on anticoagulant medication (aspirin, clopidogrel, warfarin) at the time of blood draw, but no significant difference in functional analyses was identified between groups in the types or doses of anti-platelet or anticoagulation medications.

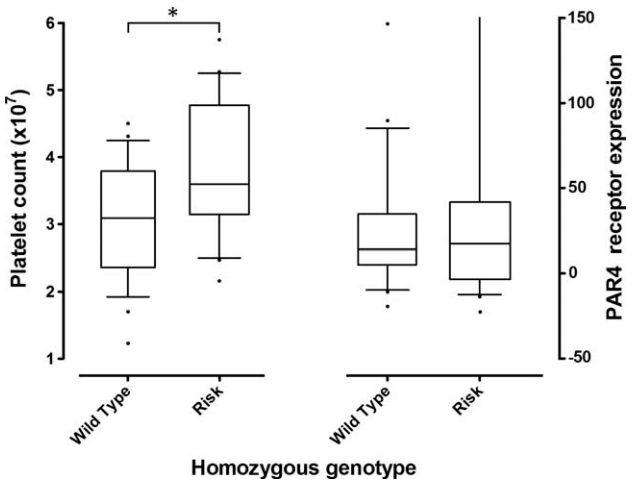

Figure 1. rs773857 genotype versus platelet count and PAR4 receptor expression. Shown are box-whisper plots of the median platelet count and PAR4 receptor expression with inter-quartile range and outliers of 23 patients with the *F2RL3* rs773857 homozygous major allele and 20 patients with the homozygous risk genotype. *P-value = 0.02.

Discussion

In this study, we report a novel association between SNP rs773857, a SNP in the region of the gene encoding the principal receptor for thrombin-mediated platelet activation PAR4, and PMI in subjects undergoing CABG surgery. Furthermore, platelets of subjects homozygous for this

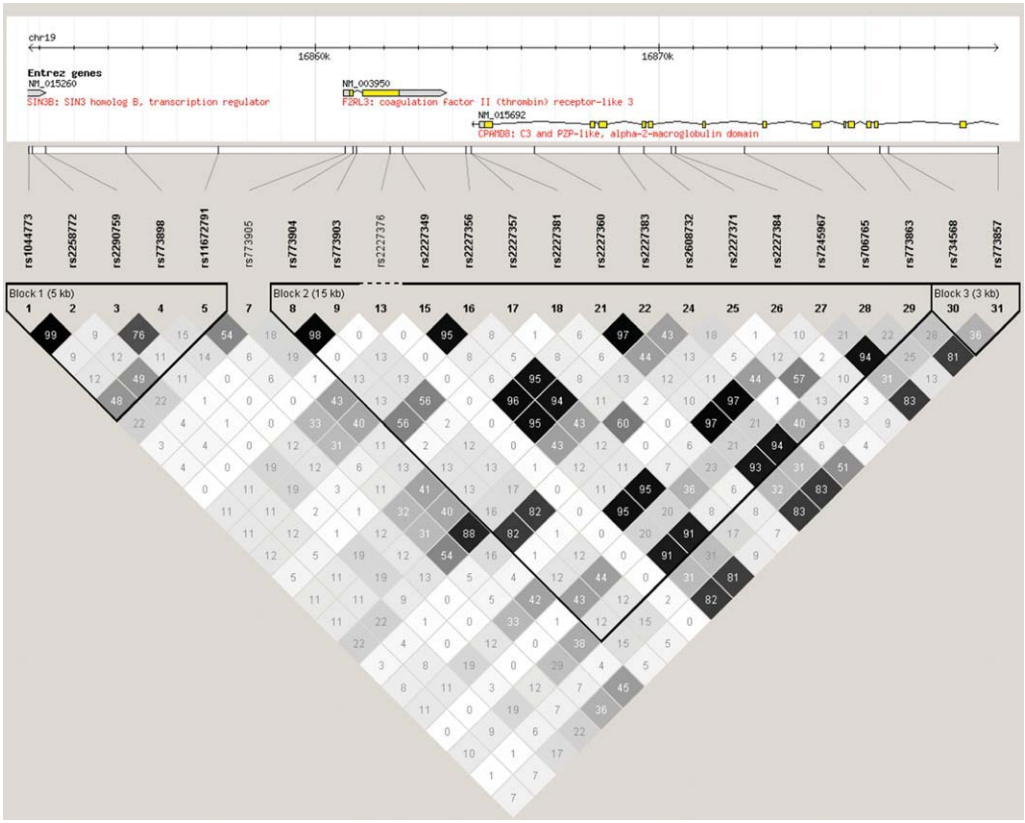

Figure 2. Linkage disequilibrium plot of *F2RL3* region. The number within each square is the correlation (r^2) between intersecting SNPs. Correlations and block structure were estimated using HaploView software (version 4.0; http://www.broad.mit.edu/mpg/haploview/). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

SNP showed a significant increase in P-selectin release after activation with a specific PAR4 agonist.

Role of thrombin in PAR activation

Both *F2R* and *F2RL3* are genes of cardiovascular relevance currently being investigated as drug targets for anticoagulation in coronary disease [31–33]. A recent study identified genome-wide significance for reduced DNA methylation in *F2RL3* in heavy smokers, suggesting a causal link between *F2RL3* and smoking-related cardiovascular pathology through its role in endothelial physiology and platelet activation [34]. Functional genetic variation has been described in the human *F2R* gene, but not in the *F2RL3* gene, although both are targets of thrombin signaling [8–10]. In rodents, PAR4 activation leads to platelet aggregation, while *F2RL3* knock-out mice display impaired coagulation, and platelets that do not respond to thrombin signaling [35]. Equally important, these *F2RL3* deficient mice are protected from thrombosis in a mesenteric arteriolar thrombosis model [35], and protected from cerebral ischemia/reperfusion injury in a transient middle cerebral artery occlusion model [36]. Rats treated with PAR4 antagonists experienced smaller infarct sizes after exposure to cardiac ischemia/reperfusion injury [37]. Of note, however, differences exist between pharmacologic inhibition (anatagonists) and *F2RL3* deficient (knock-out) mice. *F2RL3* deficient mice had larger myocardial infarcts in an ischemia/reperfusion model while pharmacologic inhibition of PAR4 proved to be cardioprotective [38].

Mechanism of action of rs773857 risk allele

The bi-exonal gene *F2RL3* for the PAR4 protein on chromosome 19 encompasses 64 SNPs, eight of which are in coding exons (Build 37.1, GRCh37) [25]. Twenty-three SNPs that encompassed three haplotypes in and up to 9 kb around the gene were genotyped. rs773857 has a minor allele frequency of 35% in Caucasians, and although located in *CPAMD8*, 17 kb downstream from the closest coding exon in *F2RL3*, has high LD to SNPs in *F2RL3* (r^2 up to 0.82) (Fig. 2). *CPAMD8* is a large gene encompassing 130 kb and 40 exons expressed on platelets and in cardiac tissue. The last five exons of *CPAMD8* overlap with the last exon and the 3'-UTR region of *F2RL3*. It is therefore plausible, that our SNP of interest is in LD with a hereto-unidentified functional variant in *F2RL3* responsible for increased thrombin receptor binding and platelet activation. Only two of the 23 genotyped SNPs are non-synonymous and upon further examination in SIFT (http://sift.jcvi.org/) [39] and SNPnexus [40], tools to examine the possible effects on the transcriptome and protein level, no damaging amino acid changes were identified.

rs773857 is located within a 5 kb LD block which contains five c-Myc transcription factor binding sites (TFBS) and 12 CTCF TFBS from ChIP-seq peaks on H1 human embryonic stem cells (Open Chromatin TFBS by ChIP-seq from ENCODE/Open Chrom (UT Austin) through the ENCODE Jan 2011 Freeze and CTCF Binding Sites by ChIP-seq from ENCODE/University of Washington) [41]. The proto-oncogene C-myc encodes for a transcription factor that regulates expression of up to 15% of genes through enhancer box binding recruitment of histone acetyltransferases [42]. Myc also regulates chromatin structure by regulating histone acetylation in gene-rich areas and in regions far from known genes [43]. The transcriptional repressor CTCF, also known as chicken 11-Zn-finger transcription factor termed CCCTC-binding factor (CTCF), is an insulator protein with an extensive role in gene regulation [44]. CTCF can block the interaction between enhancers and promoters, often as a long-range regulatory element [45]. Thus, it is conceivable that the association with PMI and the effect on platelet activation is mediated through TFBS.

Limitations

These findings have not been validated in a separate cardiac surgical cohort or a non-surgical population. Even so, this study spans two institutions and multiple surgeons, and our subjects make up a homogenous Caucasian population.

The SNP rs773857 is located within an intron of the gene *CPAMD8*, which is expressed on platelets and in cardiac tissue. Given the location of the SNP, the association with PMI could be attributed to *CPAMD8*. However, platelet activation with the specific PAR4 agonist resulting in an increased α-granule release in patients with the rs773857 risk allele makes this unlikely.

Conclusion

We identified a novel association between the SNP rs773857, in proximity to the *F2RL3* gene and also the principal receptor (PAR4) for thrombin-mediated platelet activation, and PMI in subjects undergoing CABG surgery. Patients homozygous for this SNP had increased platelet counts and their platelets showed a significant increase in α-granule release after activation with a specific PAR4 agonist.

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