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Quantitative trait locus analysis for hemostasis and thrombosis

Qila Sa,
Department of Molecular Cardiology, Joseph J. Jacobs Center for Thrombosis and Vascular Biology, Cleveland, OH 44195, USA

Department of Cardiovascular Medicine, Cleveland Clinic Lerner Research Institute, Cleveland, OH 44195, USA

Erika Hart,
Department of Molecular Cardiology, Joseph J. Jacobs Center for Thrombosis and Vascular Biology, Cleveland, OH 44195, USA

Department of Cardiovascular Medicine, Cleveland Clinic Lerner Research Institute, Cleveland, OH 44195, USA

Annie E. Hill,
Department of Genetics, Case Western Reserve University School of Medicine, Cleveland, OH 44106, USA

Joseph H. Nadeau, and
Department of Genetics, Case Western Reserve University School of Medicine, Cleveland, OH 44106, USA

Jane L. Hoover-Plow
Department of Molecular Cardiology, Joseph J. Jacobs Center for Thrombosis and Vascular Biology, Cleveland, OH 44195, USA

Department of Cardiovascular Medicine, Cleveland Clinic Lerner Research Institute, Cleveland, OH 44195, USA

Department of Molecular Cardiology, NB50, Cleveland Clinic Lerner Research Institute, Cleveland, OH 44195, USA hooverj@ccf.org

Abstract

Susceptibility to thrombosis varies in human populations as well as many in inbred mouse strains. The objective of this study was to characterize the genetic control of thrombotic risk on three chromosomes. Previously, utilizing a tail-bleeding/rebleeding assay as a surrogate of hemostasis and thrombosis function, three mouse chromosome substitution strains (CSS) (B6-Chr5$^{A/J}$, Chr11$^{A/J}$, Chr17$^{A/J}$) were identified (*Hmtb1, Hmtb2, Hmtb3*). The tailbleeding/rebleeding assay is widely used and distinguishes mice with genetic defects in blood clot formation or dissolution. In the present study, quantitative trait locus (QTL) analysis revealed a significant locus for rebleeding (clot stability) time (time between cessation of initial bleeding and start of the second bleeding) on chromosome 5, suggestive loci for bleeding time (time between start of bleeding and cessation of bleeding) also on chromosomes 5, and two suggestive loci for clot stability on chromosome 17 and one on chromosome 11. The three CSS and the parent A/J had elevated clot stability time. There was no interaction of genes on chromosome 11 with genes on chromosome 5 or chromosome 17. On chromosome 17, twenty-three candidate genes were identified in synteny

Correspondence to: Jane L. Hoover-Plow.

with previously identified loci for thrombotic risk on human chromosome 18. Thus, we have identified new QTLs and candidate genes not previously known to influence thrombotic risk.

Introduction

Thrombosis plays a critical role in the development of cardiovascular diseases (Sturm 2004) and may be caused by the upregulation of the procoagulant pathway, or the downregulation of anticoagulant and fibrinolytic pathways (Mackman 2005). Thrombotic risk has been associated with genetic variation in these three pathways (Grant 2003; Williams and Bray 2001). Mutations may directly alter components of these pathways (Hong and Kwaan 1999; Zoller et al. 1997) or may indirectly alter their regulation or the proteins required for their processing (Zhang and Ginsburg 2004). Family history has long been associated with susceptibility to thrombosis, but these known mutations account for only a small portion of the variation. The genetic complexity of human populations and the huge influence of environmental factors on phenotypes make finding genes that may have small effects on thrombotic risk a difficult task.

Mouse models have been used to determine the function of individual genes and uncover new genes underlying complex diseases such as atherosclerosis and obesity. To date, only a few studies (Lemmerhirt et al. 2007; Mohlke et al. 1996) have reported the use of quantitative trait locus (QTL) mapping in mouse models to characterize the genetic control of thrombotic risk. The two inbred mouse strains, C57BL/6J (B6) and A/J, differ markedly in susceptibility to thrombosis and fibrinolysis and are ideally suited to identify genes associated with thrombotic risk. Previously, we reported that the arterial occlusion time in the ferric chloride-induced vascular injury model in the A/J mice was twofold less than in B6 mice, and clot stability time in a tailbleeding/rebleeding assay was threefold longer in the A/J mice (Hoover-Plow et al. 2006). A panel of chromosome substitution strains (CSS; B6-Chr1-19, X, $Y^{A/J}$), with an individual A/J chromosome in a B6 background, was utilized for this study. This approach has advantages over genome-wide scans, including detection of more QTLs, the requirement of fewer mice, and simplification of subsequent fine-mapping. The panel was screened with the bleeding/rebleeding assay as a surrogate marker of thrombosis and hemostasis. The bleeding/rebleeding assay has been used to identify functional changes in hemostasis and thrombosis in several genetically engineered mice (Broze et al. 2001; Hamilton et al. 2004; Kato et al. 2004; Sweeney et al. 1990). We demonstrated that the assay reports on platelet defects and fibrinolytic component deficiencies and corresponds well with a carotid injury assay (Hoover-Plow et al. 2006). Although bleeding time was not different between A/J and B6 mice (Hoover-Plow et al. 2006), a reduced bleeding time was identified in five of the CSSs, including strains with A/J chromosomes 5, 6, 8, 14, 15, and Y. In addition, three CSS with A/J chromosomes 5, 11, and 17 were identified with increased clot stability time that was similar to the elevated values of the A/J parent strain compared to the B6 strain. The purpose of this study was to characterize the genetic control of bleeding and clot stability time on chromosomes 5, 11, and 17.

Materials and methods

Mice

The inbred mouse strains C57BL/6J (B6, #000664) and A/J (#000646) were purchased from The Jackson Laboratory (Bar Harbor, ME). The CSSs were previously described (Nadeau et al. 2000; Singer et al. 2004). CSS-5 (female), CSS-11 (female), or CSS-17 (male) were crossed with B6 to produce F_1 progeny that were intercrossed to generate F_2 progeny. Mice were housed in sterilized isolator cages with a 14-h/10-h light/dark cycle and were provided

sterilized food and water *ad libitum*. The bleeding/rebleeding assay was performed on mice (both males and females) at 6–8 weeks of age. This study was approved by the Institutional Animal Care and Use Committee and procedures were followed in accordance with institutional guidelines.

Genotyping

Genomic DNA was prepared from ear punches of the mice and genotyping was performed using polymerase chain reaction (PCR) for microsatellite markers (Mouse Mappairs, Invitrogen, Carlsbad, CA) and primers for restriction fragment length polymorphism (RFLP) markers (Operon, Huntsville, AL). PCR was performed using HotstarTaq Master Mix Kit (Qiagen, Valencia, CA). The PCR products were detected by electrophoresis on 10% polyacrylamide gel (National Diagnostics, Atlanta, GA) or on 1.5% agarose gel after digestion with restriction endonucleases (New England Biolabs, Beverly, MA) and visualized by ethidium bromide staining. Markers were selected 10–15 cM apart on each chromosome, and markers that clearly distinguished A/J and B6 genotypes were selected. The location of these markers is identified in Table 1 and Table 2.

Phenotyping

Phenotyping was performed using the bleeding/rebleeding assay as previously described (Hoover-Plow et al. 2006). Briefly, mice were anesthetized, and prewarmed tails were clipped and placed in saline. Bleeding time was measured as the time between the start of the bleeding and cessation of the bleeding. Clot stability time was measured as the time between the cessation of the bleeding and the start of the second bleeding.

Statistics

The linkage analysis was performed with MapManager QTX program (Manly et al. 2001). Ten thousand permutations of the trait values were used to define significant and suggestive thresholds and corresponded to the 95th and 37th percentiles, respectively. Kruskal-Wallis nonparametric ANOVA and the Mann-Whitney test were used to determine statistical differences in bleeding and clot stability times between genotype alleles.

Results

Chromosome 5

QTL analysis was carried out in F_2 progeny ($n = 79$) from the CSS-5 × B6 intercross. CSS mapping is typically more efficient than traditional genome-wide scanning and requires fewer animals. This was illustrated by Singer et al. (2004) (see Supplementary Material) and noted in other studies (Belknap 2003; Matin et al. 1999). We estimated that QTL mapping for clot stability time using recombinant inbred F_2 intercrosses requires nearly three times more mice than using CSSs to detect the same effect. In F_2 progeny, bleeding/clot stability times were measured and a chromosome 5 genome scan was performed. For clot stability time, a significant locus named *Hmtb4* (hemostasis thrombosis 4) was obtained at marker *D5Mit338* (59 cM) with a LOD score of 3.1 (significant threshold = 2.4) and $p = 0.0009$; $p = 0.008$ with Bonferroni correction (Bland and Altman 1995) (Fig. 1a). This locus explained 16% of the variance in clot stability time in the F_2 mice (Table 3). When clot stability time was plotted according to the genotypes at *D5Mit338*, a significant 2.9-fold increase ($p = 0.04$) was found for F_2 mice homozygous for the B6 allele compared to the F_2 mice homozygous for the A/J allele (Fig. 2a). This was unexpected because the B6 parental strain

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had a shorter clot stability time than A/J or CSS-5 mice (Hoover-Plow et al. 2006) (see Supplementary Material). Linkage analysis was also performed for bleeding time (Fig. 1b) for chromosome 5 and a suggestive peak was identified at marker *D5Mit320* (70 cM) with a LOD score of 1.5 (suggestive threshold = 0.9, significant threshold = 2.4), which accounted for 8% of the variance (Table 3). This QTL was designated as *Hmtb5*. Mice with the homozygous B6 allele had a 2.5-fold longer bleeding time compared with mice with the A/J homozygous allele (Fig. 2b). There was no gender difference ($p > 0.05$) in the parent strains, CSS mice, and F_2 progeny for clot stability and bleeding (data not shown). The F_1 mice from the crosses of CSS-5 with B6 or CSS-17 with B6 had short clot stability time similar to the B6 mice (Hoover-Plow et al. 2006). However, the F_1 mice from the cross of CSS-5 with CSS-17 conferred long clot stability time similar to A/J mice, indicating interactions between chromosome 5 and chromosome 17 (see Supplementary Material).

Chromosome 11

A CSS-11 strain that had only the A/J-derived segments from *D11Mit70* (0 cM) to *D11Mit20* (20 cM) and from *D11Mit4* (37 cM) to *D11Mit336* (75 cM) was used for the chromosome 11 QTL analysis ($n = 76$). Using F_2 mice, a suggestive locus, named *Hmtb6*, for clot stability time (Fig. 1c) was identified at marker *D11Mit336* (75 cM) with LOD score of 1.7 (suggestive threshold = 1.0, significant threshold = 4.3), explaining 10% of the variance in clot stability time (Table 3). Clot stability time of the mice with the homozygous BB genotype at the marker *D11Mit336* (Fig. 2c) was similar to the parental B6 strain (see Supplementary Material). However, the clot stability time of the mice with the heterozygous BA genotype (Fig. 2c) was significantly ($p < 0.05$) longer than the mice with the homozygous B6 genotype. The longer clot stability time in the heterozygous mice suggests overdominance (Smith et al. 2006). No gender difference was found in clot stability time in the F_2 mice. Unlike the F_1 mice from the cross CSS-5 × CSS-17, the clot stability times of the F_1 mice from the crosses of CSS-11 with CSS-5 (264 ± 48 sec, $n = 16$) or CSS-17 (320 ± 63 sec, $n = 15$) were not different than the value for B6 mice, suggesting no interactions of chromosome 11 with chromosome 5 or chromosome 17.

Chromosome 17

QTL analysis was performed in F_2 mice ($n = 130$) from the CSS-17 × B6 intercross (Fig. 1d). For clot stability time, two suggestive loci were identified. One, named *Hmtb8*, was at marker *D17Mit20* (34.3 cM) with a LOD score of 1.7 (suggestive threshold = 0.8, significant threshold = 2.3), which explained 6% of the variance (Table 3). Another locus (*Hmtb9*) for clot stability was identified at marker *D17Mit39* (45.3 cM) with a LOD score of 1.2 (suggestive threshold = 0.8, significant threshold = 2.3) that explained 4% of the variation (Table 3). As with QTLs on chromosome 5, at both *Hmtb8* and *Hmtb9* the homozygous B6 genotype conferred a longer clot stability time (Fig. 2d, e) than for the homozygous A/J genotype. This was unexpected since the A/J strain has prolonged clot stability time. No gender difference in bleeding and clot stability time was found in the F_2 mice. The QTL interval on chromosome 17 is in synteny with a human QTL for protein C resistance (Hasstedt et al. 1998;Soria et al. 2003) on the short arm of chromosome 18. This conserved region is 6.4 Mb and 23 homologous genes were identified.

Discussion

In this study we used CSSs derived from A/J and B6 inbred strains to study the genetic control of thrombosis. Specific CSSs have been shown to have many phenotypic differences in response to vascular injury (Hoover-Plow et al. 2006) and marked differences in a tail-bleeding/rebleeding assay. We identified four QTLs for clot stability on chromosomes 5, 11, and 17, and one for bleeding on chromosomes 5. These five QTLs account for 44% of the

total variance in the bleeding and clot stability phenotype, suggesting that other QTLs determining thrombotic risk remain to be identified. In this study, in addition to the five QTLs, 23 candidate genes, not previously suggested as thrombotic risk factors, were identified in the syntenic region in the *Hmtb8* QTL for clot stability on chromosome 17.

In a previous study (Hoover-Plow et al. 2006), the observation was made of the possible interaction of chromosome 5 and chromosome 17. The F_1 mice heterosomic for either chromosome 5 or chromosome 17 had similar rebleeding times compared to the B6 parental strain. Nevertheless, the F_1 mice heterosomic for both chromosome 5 and chromosome 17 conferred prolonged rebleeding time similar to the A/J parental strain, suggesting that gene interactions between the two chromosomes had an additive effect. In contrast, no interactions of chromosome 11 with chromosome 5 or chromosome 17 were found. F_1 mice from the crosses (CSS-11 × CSS-5) or (CSS-11 × CSS-17) had short clot stability time similar to the F_1 mice hetersomic for chromosome 5 or chromosome 17.

The *Hmtb4*, *Hmtb8*, and *Hmtb9* B6 alleles had prolonged clot stability times. A similar phenomenon has also been found in other studies that investigated atherosclerosis susceptibility in mice (Dansky et al. 2002; Ishimori et al. 2004). The prolonged times in our study did not coincide with phenotypes in the parental strains and suggest two possibilities: The parental strains bring a composite of allelic variants with contrasting and independent effects, or are from gene interactions. This paradox was present only in the loci on the two interacting chromosomes, chromosome 5 and chromosome 17, but not chromosome 11. A gene-gene interaction is supported by F_1 data from different crosses. Chromosome 17 carries two loci for the clot stability trait, *Hmtb8* and *Hmtb9*, and while clot stability time was recessive in F_1 for chromosome 17, both of the two loci had dominant effects in F_2. This could be explained by an inhibitory interaction between the two loci on chromosome 17 and suggests that A/J *Hmtb8* or A/J *Hmtb9* contributes to the dominant effect and that A/J *Hmtb8* and A/J *Hmtb9* have an inhibitory effect on each other. As a consequence, the combination of the two loci is recessive. Our study suggests the complexity of thrombosis and hemostasis.

In the Mouse Genome Database (2007) there are over 300 genes listed in the chromosome 5 QTL, so the next step is to generate a congenic strain with the QTL region and perform fine mapping (Armstrong et al. 2006; Christians and Keightley 2004; Wang et al. 2007). Interestingly, one QTL (*Mvwf*) associated with plasma von Willebrand factor (vWF) level was previously identified at the distal region of mouse chromosome 11 (Mohlke et al. 1996). The *Mvwf* candidate interval is between *Ngfr* and *Hoxb9* at 56 cM within the clot stability locus *Hmtb6*. Whether the *Mvwf* locus and QTL for clot stability identified in this study are related remains to be determined. In addition, four genes known to modify thrombosis are located on chromosomes 5, 11, and 17: *Serpin1* (plasminogen activator inhibitor-1) on chromosome 5; *Serpinf2* (alpha-2 antiplasmin) on chromosome 17; *Plg* (plasminogen) on chromosome 17; and *Mcfd2* (multiple coagulation deficiency 2) on chromosome 17. All four of these genes are outside the 95% CI of the respective locus suggesting that these genes are not the causative genes for the bleeding/clot stability trait.

A QTL on human chromosome 18 was reported to influence protein C resistance and thrombotic risk (Hasstedt et al. 1998; Soria et al. 2003). This human QTL region (18p11.32-11.23) coincides with the mouse QTL region on chromosome 17. Whether these two QTLs in different species result from variation of the same causative gene(s) for thrombosis remains to be verified. Twenty-three candidate genes are located in the conserved chromosome segment.

Susceptibility to thrombosis is a major risk factor for cardiovascular disease (CVD) and family history has been long associated with this risk, but the genetic determinants of thrombotic risk identified thus far do not account for the observed variation in human populations. To our knowledge, this is the first study to use CSSs for mapping QTLs for thrombosis susceptibility. Identifying the causative genes in these mouse QTLs could lead to the identification of new thrombotic risk factors in humans and ultimately to new therapeutic approaches to prevent and treat thrombosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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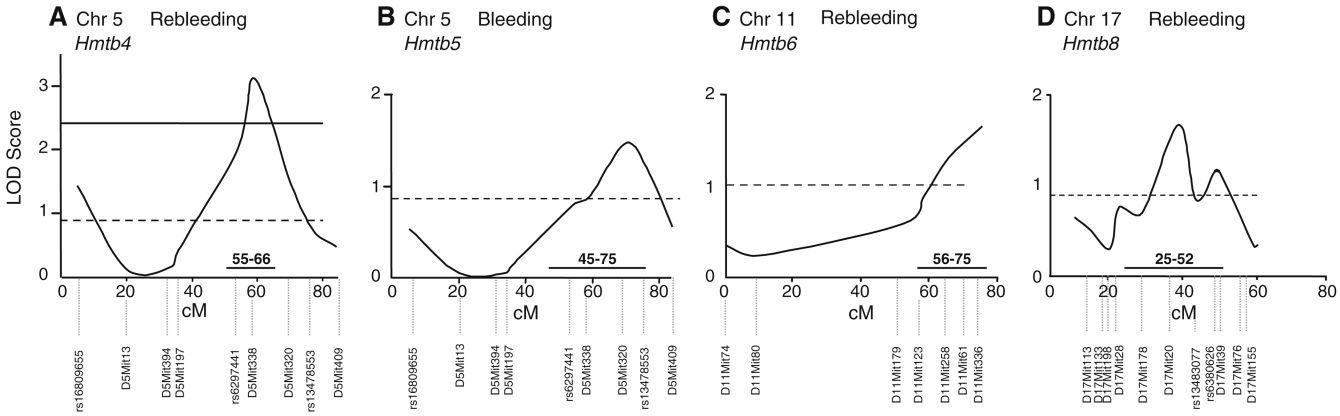

Fig. 1.

QTL analysis. (**a**) *Hmtb4*, peak marker *D5Mit338* (59 cM). (**b**) *Hmtb5*, peak marker *D5Mit320* (70 cM). (**c**) *Hmtb6*, peak marker *D11Mit336* (75 cM). (**d**) *Hmtb8*, peak marker *D17Mit* 20 (34.3 cM); *Hmtb9*, peak marker *D17Mit39* (45.3 cM). The linkage analysis was performed with MapManager QTX program. (Manly et al. 2001). Solid line indicates significant threshold (0.05). Dashed line indicates suggestive threshold (0.63). The 95% confidence intervals for each QTL are indicated

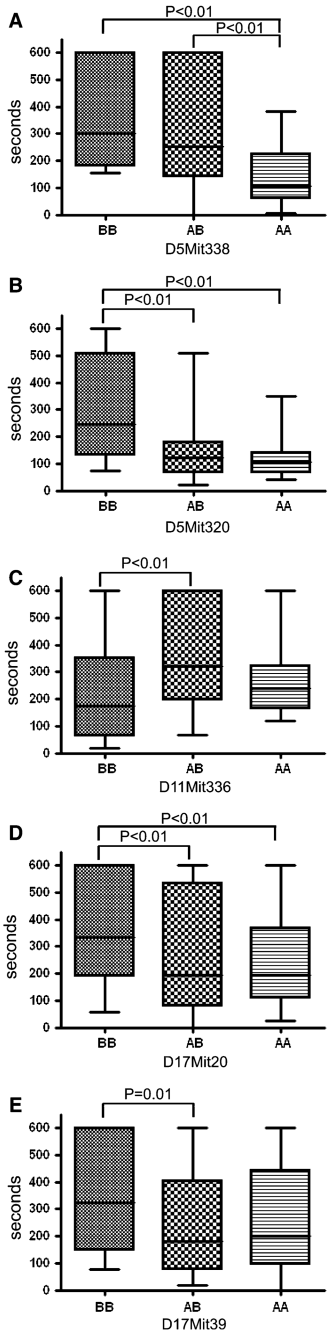

Fig. 2.

The allele distributions at peak markers in the F_2 mice. (**a**) *Hmtb4*, clot stability time, chromosome 5, *D5Mit338* BB, *n* = 14; AB, 45; AA, 20. (**b**) *Hmtb5*, bleeding time, chromosome 5, *D5Mit320* BB, *n* = 10; AB, 47; AA, 22. (**c**) *Hmtb6*, clot stability time, chromosome 11, *D11Mit336* BB, *n* = 27; AB, 34; AA, 15. (**d**) *Hmtb8*, clot stability time, chromosome 17, *D17Mit20* BB, *n* = 39; AB, 60; AA, 31. (**e**) *Hmtb9*, clot stability time, chromosome 17, *D17Mit39* BB, *n* = 35; AB, 48; AA, 47. A = A/J; B = B6. The lines at the middle indicate the medians. The boxes show the 25th and 75th percentiles. The whiskers show the ranges. Statistical differences are indicated between bars

Table 1

Microsatellite markers and their chromosomal position[a]

Chromosome 5			Chromosome 11			Chromosome 17		
Marker	**Position (cM)**	**Position (Mb)**	**Marker**	**Position (cM)**	**Position (Mb)**	**Marker**	**Position (cM)**	**Position (Mb)**
rs16809655	5.0	20.3	*D11Mit74*	0	0.5	*D17Mit113*	6.5	12.0
D5Mit13	20	35.9	*D11Mit80*	10	20.0	*D17Mit133*	10.4	24.5
D5Mit394	34	53.2	*D11Mit179*	52	90.0	*D17Mit198*	16	27.5
D5Mit197	36	63.4	*D11Mit123*	58	100.0	*D17Mit28*	18.4	33.5
rs6297441	54	98.0	*D11Mit258*	65	107.5	*D17Mit178*	24.5	48.0
D5Mit338	59	107.7	*D11Mit61*	70	110.5	*D17Mit20*	34.3	57.0
D5Mit320	70	125.5	*D11Mit336*	75	112.0	*rs13483077*	39.0	64.8
rs13478553	77	135.7				*rs6380626*	44.0	72.0
D5Mit409	84	144.8				*D17Mit39*	45.3	74.0
						D17Mit76	54.6	85.5
						D17Mit155	55.7	84.5

[a]Position of markers obtained from Mouse Genome Database (Mouse Genome Database 2007)

Table 2

RFLP markers, their chromosomal positions, primers, and restriction endonucleases

SNP ID	Position (bp)	Forward primer	Reverse primer	Enzyme
rs16809655	Chr 5: 20274741	GCAACCCAGATCAAGCATAAGA	ATGATGAGAAGGTCCCCACA	*Sal*I
rs6297441	Chr 5: 98820203	TAAGGCTGGGGAATGGTTTG	GGATTGGGTCTGACAACATAGG	*Apa*LI
re13478449	Chr 5: 107393510	CCGTAGGTTCGTACCCACC	GTCCCATCATATTCCACAAAGTGC	*Eco*RV
rs13478553	Chr 5: 135724469	CATAGCCCAGCCCTCTGC	GGAGACACCACAAGCAGAATTG	*Xho*I
rs13483077	Chr 17: 64806259	GAAGGTACTGTCCCCGAGTC	TGGCGACGACTAAGCTACTT	*Xho*I
rs6380626	Chr 17: 71942738	TCCTGCTACCTCTCCTAGGAC	CTGTGAGTCTGTGTGTGGGT	*Hin*fI

Table 3

QTL analysis

Locus name	Marker	Phenotype	Position (cM)[a]	Position (Mb)	LOD score	95% CI	Variance %	*p* value[b]	Significance
Hmtb4	*D5Mit338*	Clot stability	59	107.7	3.1	55–66	16	0.0009	Significant
Hmtb5	*D5Mit320*	Bleeding	70	125.5	1.5	45–75	8	0.02	Suggestive
Hmtb6	*D11Mit336*	Clot stability	75.0	110.5	1.7	56–75	10	0.02	Suggestive
Hmtb8	*D17Mit20*	Clot stability	34.3	57.0	1.7	25–52	6	0.02	Suggestive
Hmtb9	*D17Mit39*	Clot stability	45.3	74.0	1.2	0–56	4	0.06	Suggestive

[a] From Mouse Genome Database (2007). Number of F_2 mice analyzed: chromosome 5, $n = 79$; chromosome 11, $n = 76$; chromosome 17, $n = 130$

[b] Chromosome-wide