IN FOCUS

Genome scan of venous thrombosis in a pedigree with protein C deficiency

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Summary. Kindred Vermont II has a high frequency of venous thrombosis, occurring primarily in pedigree members with type I protein C deficiency due to a 3363 inserted (Ins) C mutation in exon 6 of the protein C gene. However, only a subset of 3363 InsC carriers have suffered thrombotic episodes, suggesting that the increased risk of thrombosis results upon the co-occurrence of 3363 InsC with a second, unknown, thrombophilic mutation that segregates independently within the pedigree. To test this hypothesis and to localize the co-occurring gene, we performed a genome scan of venous thrombosis in Kindred Vermont II. Non-parametric linkage statistics identified three potential gene locations, on chromosomes 11q23 (nominal $P < 0.0001$), 18p11.2-q11.2 ($P < 0.0007$), and 10p12 ($P < 0.0003$), supporting the presence of at least one additional thrombophilic mutation in the pedigree. Identification of the unknown mutation(s) promises to reveal a new genetic risk factor for thrombophilia, contribute to our understanding of the blood clotting mechanism, and expand our knowledge of the diversity of oligogenic disease.

Keywords: genetics, linkage.

Introduction

Venous thrombosis occurs with an annual incidence of 1–2 per 1000 [1]. Common risk factors include surgery, malignancy, hyperhomocysteinemia, and high levels of procoagulant factor II, factor (F)VIII, and factor XI, as well as several genetic

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variants [2]. The high frequency of some genetic variants, in particular factor (F)V Leiden [3] and prothrombin G20210A [4], suggests oligogenic inheritance, that thrombophilia results from the co-occurrence of two or more variants [5]. Oligogenic inheritance can also explain the higher risk observed for specific variants in individuals with a positive family history [6]. In fact, oligogenic inheritance may be common, having recently been demonstrated or proposed for a number of diseases [7,8].

Less common than either FV Leiden or prothrombin G20210A, protein C deficiency, due to heterozygosity of any of a number of mutations [9], increases thrombosis risk, although most individuals with protein C deficiency are asymptomatic [10]. Likewise, in a large Vermont pedigree (Kindred Vermont II), a 3363 inserted (Ins) C mutation in exon 6 of the protein C gene [11] increases, but appears insufficient to completely explain, venous thrombosis risk [12,13].

In some protein C-deficient families, FV Leiden has been identified as the co-occurring thrombophilic mutation [14], but the presence of only four carriers rules out FV Leiden as a major contributor in Kindred Vermont II. In contrast, 37 pedigree members carry prothrombin G20210A, but again we ruled it out as the co-occurring mutation [15]. In addition, we have ruled out prostaglandin H synthase 1 [16], α_1-microglobulin/bikunin precursor (AMBP) (unpublished data), and 34 other thrombosis candidate genes [17].

As an alternative to testing candidate genes, a genome scan can localize novel genes not previously known to be involved in the disease process. Here we present a genome scan of venous thrombosis performed to identify potential regions for a genetic mutation that co-occurs with 3363 InsC protein C to increase risk of venous thrombosis in Kindred Vermont II.

Subjects and methods

The sample used in this analysis consists of a single large pedigree, Kindred Vermont II, with a high rate of venous thrombosis [12], partially attributable to type I protein C deficiency due to 3363 InsC mutation in protein C [11]. All

available descendants of a couple born in the 1800s were studied; spouses were included whenever offspring were available. The pedigree spans six generations with data available on the most recent four. Pedigree members reside primarily in Vermont and are descendants of French Canadians and Abenaki American Indians. This study was approved by the Human Experimentation Committees of the University of Vermont College of Medicine and Beth Israel Hospital, Boston.

A total of 28 diagnoses of venous thrombosis were made, each based on a self-report of hospitalization for treatment of deep vein thromboembolism and confirmation through objective tests. Median onset ages were 45 years (range 20–53 years) in 12 male and 22 years (range17–76 years) in 15 female descendants of the pedigree founders; one additional thrombotic event occurred at age 71 years in the husband of a pedigree member.

A total of 289 individuals were genotyped for 375 autosomal markers by the NHLBI Mammalian Genotyping Service at the Marshfield Medical Research Foundation [18] using Screening Set version 10. Marker spacing averaged 9.4 cM (range 0–18 cM). Marker heterozygosity averaged 75% (range 42–89%). Using Eclipse2, the pairs version of Eclipse3 [19], and multipoint information from all autosomal markers, we identified 11 probable pedigree errors. The pedigree structure was modified to reflect this information. Genotyping errors were identified from multipoint genotypes using Simwalk2 [20,21] using input files produced by MEGA2 [22]. The mistyping analysis identified 1389 genotypes ($\sim 1\%$ of the total) having error probability $\geq 25\%$; each possibly erroneous genotype was designated a missing value in all further analyses. Using the pedigree structure corrected for likely errors and marker genotypes eliminating likely errors, maximum likelihood estimates of the marker allele frequencies were obtained in PAP [23].

For the present analysis, the pedigree was reduced from 452 (289 genotyped) to 202 (132 genotyped) by eliminating individuals who were both unaffected with thrombosis and uninformative about the haplotypes of affected pedigree members. Four non-parametric linkage statistics were computed using Simwalk2 [20] using input files produced by MEGA2 [22]. These statistics, designated A–D by Sobel and Lange [20] and in the Simwalk2 documentation, are: (A) the number of distinct haplotypes among affected individuals; (B) the highest number of affected individuals with the same haplotype; (C) entropy of haplotypes among the affected individuals; (D) kinship among affected individuals. Statistic (A) is most powerful for recessive diseases; statistic (B) is most powerful for dominant diseases; Statistics (C) and (D) measure overrepresentation of a few haplotypes among affected pedigree members. Multipoint linkage statistics A–D were computed at each genome location of a genotyped marker.

Conservative nominal empiric *P*-values of each statistic were produced by Simwalk2 by comparing the values of the statistics to the unconditional distributions of the same statistics ignoring marker phenotypes in the pedigree. Genome-wide

P-values of all four statistics simultaneously were estimated through simulation. One thousand replicates of the 22 chromosomes were simulated using the method of Terwilliger *et al.* [24] as implemented in PAP [23]. Statistics A–D were computed directly from the simulated haplotypes since the computer time requirements prohibited the inference of haplotypes from simulated marker genotypes; for each statistic, the mean for all locations and replicates varied by < 1% from the mean across all locations of the expected values produced by SimWalk2. The genome-wide *P*-value was estimated as the proportion of replicates producing, for at least one location, more extreme values of all four statistics than those obtained.

Additional markers in interesting chromosomal regions were genotyped on 132 pedigree members using an ABI310 or 3100 at the Vermont Cancer Center DNA analysis facility. Each added marker was positioned on the Marshfield map using relative locations on the physical map from the National Center for Biotechnology Information (NCBI, Build 33, April 2003) assembly, the GeneLoc map (Weizmann Institute of Science), and/or the deCODE map [25]. The mistyping analyses, allele frequency estimation, and non-parametric linkage analyses, as described above, were repeated on each of the chromosomes, with the original and added markers.

Results

Figure 1 presents the genome scan of venous thrombosis. Four regions, on chromosomes 2, 10, 11, and 18, produced nominal $P < 0.01$ for at least one of the four non-parametric linkage statistics. As shown in Table 1, chromosomes 2 and 11 attained significant linkage (genome-wide *P*-value < 0.05), while chromosomes 10 and 18 attained suggestive linkage (genome-wide *P*-value < 1.0).

The region of chromosome 2 identified by the genome scan (Fig. 1 and Table 1), contains the protein C gene [26]. Significant linkage evidence on chromosome 2 was expected since 3363 InsC protein C segregates in this pedigree [11] and increases thrombosis risk [12,13]; 22 of the 28 thrombosis cases in the pedigree are heterozygous for this mutation.

Chromosome 11 attained similar genome-wide and higher nominal significance than chromosome 2 (Fig. 1 and Table 1). Because the frequencies of the thrombosis-associated D2S1334 and D11S1986 alleles were 32% and 9%, respectively, the former occurred more often than the latter on non-thrombosis-associated chromosomes of the parents of thrombosis cases, reducing the multipoint linkage information and consequently the significance of the nominal *P*-values for chromosome 2. In contrast, the assumption of full information when applied to similar values for the statistics, produced similar genome-wide *P*-values for chromosomes 2 and 11, with strong support from statistics B, C, and D, and little support from statistic A, whose strength lies with recessive diseases. The addition of 17 markers on chromosome 11 strengthened the linkage evidence (Fig. 2 and Table 2). The implicated region ranges approximately 13 cM from D11S1778 to D11S4171.
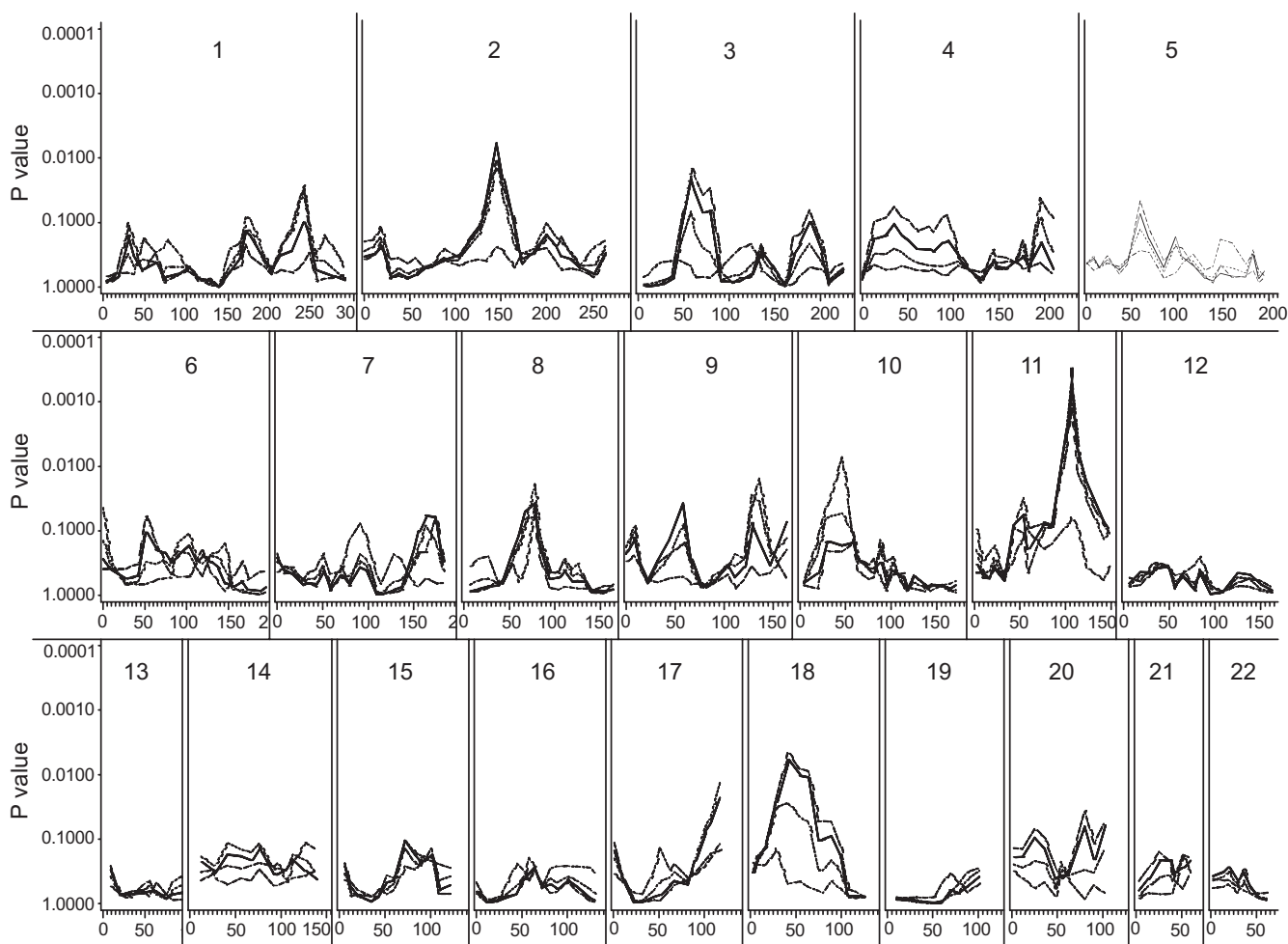
Fig. 1. Genome scan of venous thrombosis. Shown are *P*-values for non-parametric linkage statistics A (irregular), B (dashed), C (dotted), and D (solid) by centimorgan (cM) based on the Marshfield map for the 22 autosomes. See Subjects and methods for definitions of the statistics.

Table 1 Genome locations with nominal $P < 0.01$ for at least one non-parametric linkage statistic

			P-value, nominal by linkage statistic†				
Chromosome	Location* (cM)	Marker	A	B	C	D	*P*-value, genome-wide†
2	145	D2S1334	0.2413	0.0112	0.0110	0.0061	0.046
10	46	D10S1423	0.0074	0.2133	0.0549	0.1710	0.668
11	106	D11S1986	0.0634	0.0010	0.0006	0.0003	0.043
18	41	D18S542	0.4993	0.0047	0.0282	0.0061	0.434

*Marshfield map. †See Subjects and methods for description.

Chromosome 18 showed weaker linkage evidence than did either chromosome 2 or 11 with a genome-wide *P*-value in the suggestive range (Fig. 1 and Table 1). Again, support from statistics B, C, and D, but not A, suggests dominant rather than recessive inheritance. The addition of 23 markers to chromosome 18 strengthened the linkage evidence (Fig. 3 and Table 2). The implicated region ranges about 24 cM across the centromere from D18S542 to D18S877.

Chromosome 10 also showed genome-wide linkage evidence in the suggestive range (Fig. 1 and Table 1). In contrast to the other three regions, highest significance was attained for statistic A, which is most powerful for recessive inheritance. The addition of 14 markers to chromosome 10 strengthened the evidence produced by all the statistics (Fig. 4 and Table 2). The implicated region ranges about 18 cM, from D10S1430 to D10S1789.

Discussion

The 3363 InsC protein C mutation increases the risk of venous thrombosis in Kindred Vermont II [11]. Nevertheless, only 15 of 39 mutation carriers over age 40 years have experienced a
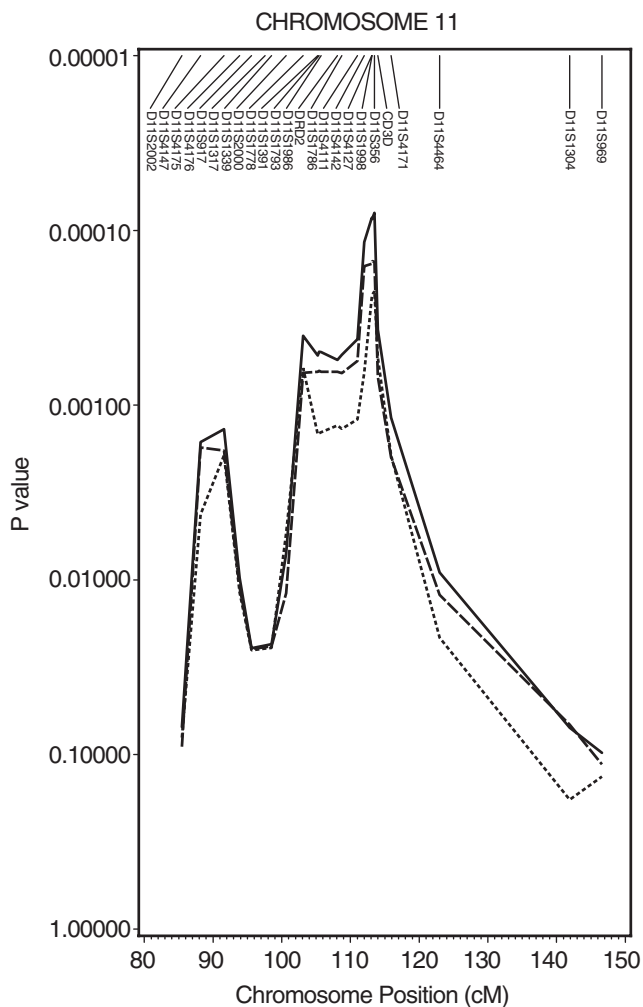
Fig. 2. Fine-mapping of chromosome 11. Shown are *P*-values for statistics B (long dashed), C (short dashed), and D (solid) by centimorgan (cM) across the region identified in the genome scan.

Table 2 *P*-values for each genome location after fine-mapping

Chromosome	Location* (cM)	Marker	Nominal *P*-value by linkage statistic†			
			A	B	C	D
10	45	D10S1125	0.0003	0.0479	0.0047	0.0315
11	113	D11S1998	0.0687	0.0002	0.0002	0.0001
18	38	D18S1158	0.4237	0.0007	0.0032	0.0007

*Marshfield map. †See Subjects and methods for description.

thrombotic episode, leading us to hypothesize that a second thrombophilic mutation segregates independently within Kindred Vermont II, and that most thrombotic episodes in pedigree members can be attributed to the co-occurrence of an unknown mutation with protein C deficiency [13]. This genome scan provides three strong candidate regions for the unknown thrombophilic gene.

Of the three regions, chromosome 11q23 attained the highest significance both before and after finemapping. A shared

chromosome 11 haplotype was inferred for 18 thrombosis cases, compared with 17 for the protein C region (where the true number is 22). This suggests a risk from a chromosome 11 gene similar in magnitude to the risk from 3363 InsC. An interesting candidate gene located within this region is the β subunit of platelet-activating factor acetylhydrolase (PAF-AH1B2) located at 11q23 [27]. PAF-AH1B2 downregulates PAF at the cell surface [28] and thus, if deficient, leads to enhanced inflammatory response at the vascular wall with likely increased local clotting activity. Dysregulation of PAF by the plasma form of PAFAH located on chromosome 6p21 has been associated with thrombotic and inflammatory disease [28].

A second candidate region, chromosome 18p11.2-q11.2, provides a credible alternative to chromosome 11q23. Despite lower significance, 18 thrombosis cases were again inferred to share a haplotype. Additional support for the region comes from linkage of activated protein C resistance and, pleiotropically, FVIII and thrombosis in the Genetic Analysis of Idiopathic Thrombophilia (GAIT) project [29]; their log of the odds (lod) score peaked at D18S53, 2.3 cM on the Marshfield map from our linkage peak. Unfortunately, the chromosome 18 region contains no obvious candidate genes.

The third candidate region, chromosome 10p12, was identified by statistic A, which is most powerful for recessive diseases. In contrast, both chromosomes 11q23 and 18p11.1-q11.2 were identified by statistics most powerful for dominant inheritance, thereby agreeing with our previous model-fitting [13]. Nevertheless, additional support for chromosome 10p12 derives from linkage of factor (F)XII in the GAIT project [30]; their lod score peaked at D10S1653, 5.3 cM on the Marshfield map from our linkage peak. Their failure to find a corresponding chromosome 10p12 effect on thrombosis risk may be due to the absence in the GAIT pedigrees of an appropriate co-occurring mutation, such as in protein C; FXII deficiency increases risk of venous thrombosis [31].

Based on earlier study of a pedigree subset, chromosome 9q33 was our strongest candidate region. Consequently, we tested and rejected as candidates prostaglandin H synthase 1 (PTGS1) [16] and α₁-microglobulin/bikunin precursor (AMBP) (unpublished data). The present analysis produced suggestive linkage evidence (genome-wide *P* < 0.812) on chromosome 9q33. Adding PTGS1, AMBP, and ABO to the chromosome 9 map failed to strengthen the weak linkage evidence (nominal *P* = 0.016 before and *P* = 0.019 after adding markers), eliminating chromosome 9q33 from further consideration.

Of the three other candidate regions, we expect that two are false positives, and that the thrombosis cases in the pedigree result primarily from a single additional mutation that co-occurs with protein C deficiency based on the fit of the data to that model [13]. However, each of the three candidate regions has its strengths. The *P*-values most strongly support chromosome 11q23, but chromosome 18p11.2-q11.2 attained an equivalent value of statistic B, and is supported by another study. The recessive inheritance suggested by the chromosome 10p12 linkage was unexpected, but a chromosome 10 variant as common as other thrombophilic variants and/or undetected
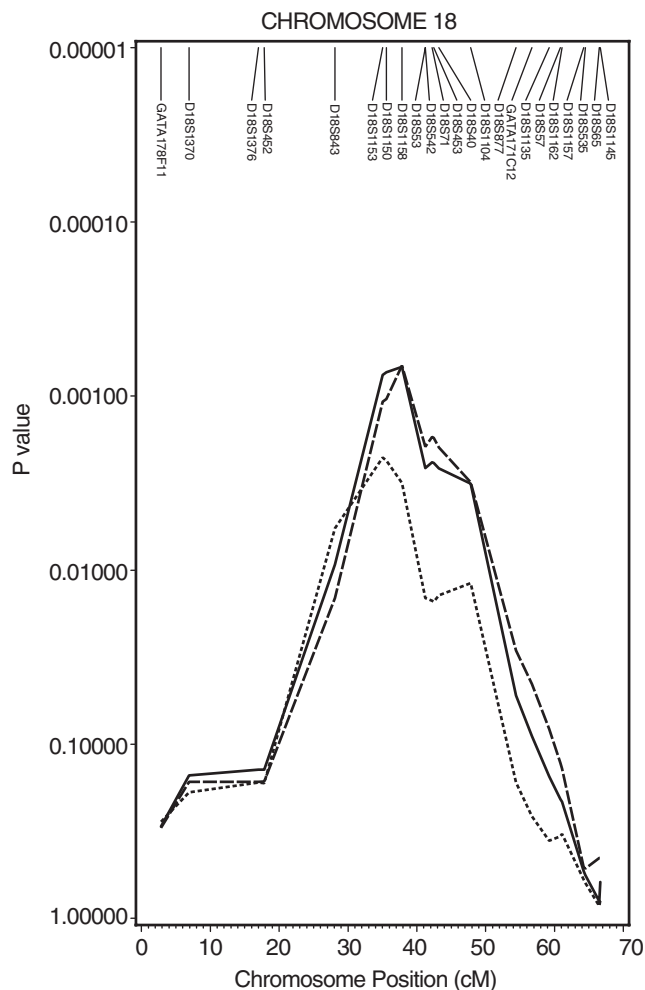
Fig. 3. Fine-mapping of chromosome 18. Shown are *P*-values for statistics B (long dashed), C (short dashed), and D (solid) by centimorgan (cM) across the region identified in the genome scan.

Fig. 4. Fine-mapping of chromosome 10. Shown are *P*-values for statistics A (irregular), B (long dashed), C (short dashed), and D (solid) by centimorgan (cM) across the region identified in the genome scan.

inbreeding might allow the clustering of multiple copies of an allele within a pedigree. Alternatively, more than one additional mutation may co-occur with protein C deficiency in this pedigree. Since the dissection of the genes underlying oligogenic diseases is in its infancy, characteristics of those genes remain to be discovered.

Despite resulting from a single pedigree, we expect any findings from this sample to apply to the population at large. In fact, 3363 InsC protein C is common among protein C-deficient individuals in France and spread widely in Quebec upon introduction by an immigrant French couple who settled near Quebec City in 1669 [32]. Although the unknown mutation may increase thrombosis risk only upon co-occurrence with 3363 InsC or only upon co-occurrence with any of the many mutations that cause type I protein C deficiency [9], it probably increases thrombosis risk upon co-occurrence with any other thrombophilic mutation. If common, its discovery could lead to an important new risk factor for thrombosis. Even if rare, it promises to yield insight into the blood-clotting
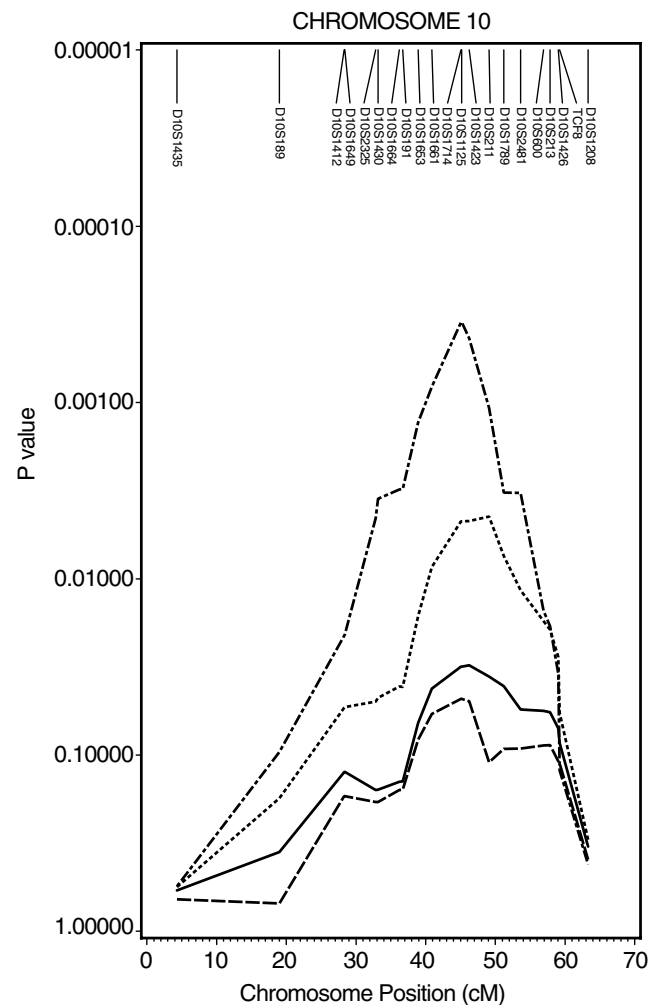
mechanism. By limiting the sample to a single pedigree, we simplified the well-documented complexity of thrombophilia, obtained a large number of individuals with the identical mutation, and increased the chances of only a single additional thrombophilic mutation.

In summary, a genome scan has produced three candidate regions for a gene that co-occurs with protein C deficiency to increase the risk of venous thrombosis in a single large pedigree. The identification of the gene and its mutation will increase understanding of the blood-clotting mechanism and also presents an opportunity to understand a oligogenic disease.

Acknowledgements

Electronic database information

URLs for data presented herein are as follows:

GeneLoc: http://genecards.weizman.ac.il/

Marshfield Linkage Maps: http://research.marshfieldclinic.org/genetics/

Mega2: http://watson.hgen.pitt.edu/mega2.html

NCBI: http://www.ncbi.nlm.nih.gov

PAP: http://hasstedt.utah.edu/

SimWalk2: http://www.genetics.ucla.edu/software/simwalk2.html

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