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Co-segregation of the PROS1 locus and protein S deficiency in families having no detectable mutations in PROS1

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Summary. Inherited deficiency of protein S constitutes an important risk factor of venous thrombosis. Many reports have demonstrated that causative mutations in the protein S gene are found only in approximately 50% of the cases with protein S deficiency. It is uncertain whether the protein S gene is causative in all cases of protein S deficiency or if other genes are involved in cases where no mutation is identified. The aim of the current study was to determine whether haplotypes of the protein S gene cosegregate with the disease phenotype in cases where no mutations have been found. Eight protein S-deficient families comprising 115 individuals where previous DNA sequencing had failed to detect any causative mutations were analyzed using four microsatellite markers in the protein S gene region. Co-segregation between microsatellite haplotypes and protein S deficiency was found in seven of the investigated families, one family being uninformative. This suggests that the causative genetic defects are located in or close to the protein S gene in a majority of such cases where no mutations have been found.

Keywords: coagulation, PROS1, protein C, protein S, vitamin K, thrombosis.

Introduction

Protein S (PS) is a vitamin K-dependent single-chain plasma protein that regulates blood coagulation. Its main function is to serve as a nonenzymatic cofactor to activated protein C (APC) in the proteolytic degradation of procoagulant factors Va and VIIIa [1]. Inherited deficiency of PS is an autosomal dominant disorder that is associated with venous thrombosis. Homozygous or compound heterozygous deficiency of PS is extremely rare. Heterozygous PS deficiency is present in approximately 2–5% of thrombosis patients [2,3], whereas the prevalence in the general population has been estimated to be between 0.03 and 0.13% [4]. In plasma, PS circulates both as free protein and in complex with C4b-binding protein. Approximately 30% of PS is found in the free form and it is only the free form that can function as a cofactor to APC [5]. Due to the different forms of PS, three types of PS deficiency have been proposed. Type I is characterized by low levels of both total and free PS. Type II entails diminished activity of PS but normal levels of free and total PS. Type III deficiency is characterized by a low level of free PS and a normal level of total PS. Type I and III deficiency have been shown to coexist in many families [6–12], indicating that these two types of deficiencies are phenotypic variants of the same genetic disease. There are a number of factors that influence the plasma level of PS, including the genetic [13] and physiologic status, which lead to an overlap in PS levels between healthy individuals and PS deficient patients. PS increases with age [8] and decreases during pregnancy and oral contraception [14]. Furthermore, different assays for PS are influenced by preanalytical variables and suffer from limitations in analytical performance making a correct laboratory diagnosis a difficult task [15–17]. Of the assays, a low free PS level has been found to be the most reliable way of diagnosing PS deficiency [6,18].

The gene for protein S (PROS1) is located near the centromere on chromosome 3p11.1-q11.2 [19]. It is approximately 80 kb long and contains 15 exons [19–22]. The large size of the gene, in addition to the presence of a pseudogene (PROS2), complicates genetic analysis of the PROS1 gene. Nevertheless, screening for PROS1 gene mutations has been performed in a number of studies. The proportion of cases where no mutations are detected varies widely between studies [10,17,23–33]. When all cases are pooled together, PROS1 gene mutations are not detected in 47% of PS deficient families. The reason for the low prevalence of PROS1 mutations in well-characterized PS deficient patients is unknown.

The present study investigates whether microsatellite marker haplotypes cosegregate with PS deficiency in type III or mixed type I and III PS deficient families, where mutations in the PROS1 gene despite sequencing have not been found.
Materials and methods

Patients

In total, eight unrelated type III or mixed type I and type III PS deficient families, comprising 115 individuals, with a family history of venous thrombosis were investigated (Table 1). The characteristics and the diagnostic criteria of the phenotypic validation have been described previously [6,33]. Of the 56 individuals with PS deficiency, 21 had experienced venous thrombosis. The current study uses the same family identification codes as was used in the previous studies [6,33]. A PS deficient member of each family was previously investigated by DNA-sequencing of all exons and exon–intron boundaries for the presence of mutations that could explain the PS deficiency [33]. That study included a total of 17 families. In eight of these families (47%) no mutation could be identified [33]. All of these eight families are analyzed in the current study. The ethical committee approved the study and appropriate consent was obtained from the participating individuals.

Microsatellite marker analysis

Genomic DNA was extracted from blood collected in EDTA using QIAamp DNA Blood Maxi Kit (Qiagen, Germany). DNA concentrations were determined by fluorometry using Pico Green. Four microsatellite markers, D3S2388, D3S1595, D3S1552 and D3S3634 were used for segregation analysis. The map positions of the four markers have been established by using a combination of the deCode, Marshfield and Genethon maps since neither of them includes all four markers. The four microsatellite markers were positioned in the above-mentioned maps since neither of them includes all four markers. The four microsatellite markers were positioned in the above-mentioned order with the PROS1 gene located between markers D3S1552 and D3S3634. The distances from D3S2388 to D3S1595, to D3S1552 and to D3S3634 were 1.58, 3.20 and 4.30 cM, respectively. The microsatellite markers were amplified using PCR conditions as recommended by the manufacturer of the Taq polymerase (Applied Biosystems, USA) and an annealing temperature of 58 °C for marker D3S1552 and 62 °C for the remaining three markers. PCR products were resolved using capillary electrophoresis run on an ABI PRISM™ 310 sequencer employing GeneScan software (Applied Biosystems, USA). The allele sizes of the microsatellite markers were determined in relation to a size marker. GeneScan-350™ ROX (Applied Biosystems, USA). All participating individuals were successfully genotyped for all markers; hence there were no missing values.

Genetic analysis

The pedigrees were constructed using Progeny (Progeny Software LLC, USA). Multi-point LOD-scores were calculated using Genehunter [34] and Simwalk2 [35]. Genehunter was used for families 11, 20, 26 and 31, whereas Simwalk2 was used for families 8, 13, 15 and 17, as these families were too extensive for Genehunter. Simwalk2 calculates location-score, which is fully comparable to LOD-score. The penetrance was assumed to be 100% and the frequency of the disease allele 0.001 [18]. In family number 26, the cosegregation analysis suggested incomplete penetrance and the disease status of the two healthy siblings in this family was therefore given unknown status. In family number 15 the affected family members had a null allele for D3S3634, and in family number 31 the affected family members had a null allele for both D3S1595 and D3S3634. For this reason, these markers were not used in the calculations for these families. Microsatellite marker allele frequencies were calculated from 48 unrelated individuals not affected by PS deficiency. These individuals are sampled from the same population as the investigated families. Genotype inconsistencies were identified manually and using PedCheck [36]. Repeated genotyping made it possible to correct all inconsistencies. Haplotypes for all individuals were reconstructed using Simwalk2, which also searched for recombination events.

Results

A total of 115 individuals from eight protein S deficient families were genotyped using four microsatellite markers from the

<table>
<thead>
<tr>
<th>Family number</th>
<th>Number of individuals</th>
<th>Number of PS-deficient individuals*</th>
<th>Disease-associated haplotype</th>
<th>Multi-point LOD-scores</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>D3S2388</td>
</tr>
<tr>
<td>8</td>
<td>16</td>
<td>9 (4)</td>
<td>4,4,4,3</td>
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<td>6</td>
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<tr>
<td>13†</td>
<td>31</td>
<td>10 (1)</td>
<td>3,2,4,2 and 4,4,4,2</td>
<td>3.43</td>
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<td>22</td>
<td>12 (3)</td>
<td>3,8,4,–</td>
<td>3.24</td>
</tr>
<tr>
<td>17</td>
<td>20</td>
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<td>3,4,3,3</td>
<td>3.83</td>
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<td>20</td>
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<tr>
<td>26§</td>
<td>5</td>
<td>2 (2)</td>
<td>6,–,4,—</td>
<td>1.18</td>
</tr>
<tr>
<td>31§</td>
<td>7</td>
<td>5 (1)</td>
<td>6,–,4,—</td>
<td>–</td>
</tr>
</tbody>
</table>

*All families, except family 26, consist of mixed type I and type III phenotypes. The number of type III deficient individuals is given in parenthesis and is defined by low free protein S concentration and a total protein S concentration within the normal reference range [33].†Two disease-associated haplotypes identified in two branches of this family. §Null alleles present. Markers indicated by – were not used in linkage analysis. §This type III family is uninformative due to the small size of the family.
PROS1 gene region. There was cosegregation between PS deficiency and certain microsatellite haplotypes in seven informative families, family 26 being uninformative. Each family had its own unique disease-associated haplotype (Table 1). As an example, the pedigree of family number 8 is shown in Fig. 1(A). Haplotype 4, 4, 4, 3 is present in all PS deficient individuals and is absent in all healthy individuals in this pedigree. In family number 13, there were two clear divisions of the family with a certain microsatellite haplotype cosegregating with the disease for each division (Fig. 1B). The two haplotypes shared alleles for markers D3S1552 and D3S3634 (flanking the PROS1 gene), indicating that one of the haplotypes most likely has emerged from the other by recombination. All PS deficient individuals within a family shared the same haplotype and there was no case of a nonaffected member with the disease-associated haplotype, except for two possible cases: one healthy individual in family number 13 (individual IV:6, Fig. 1B) could not be excluded from having the disease-associated haplotype; and family number 26 where all four children shared the same haplotypes, yet only two of them were clearly affected by PS deficiency. However, as the father in this family was deceased, his protein S status was unknown and his DNA unavailable for analysis, the family was uninformative. Apart from these possible exceptions, no healthy individuals had a disease-associated haplotype. Thus, the penetrance was 100% in the informative families.

The pattern of cosegregation observed for all informative families is compatible with an explanation where the PROS1 gene is causative of the protein S deficiency. The strength of this conclusion at the family level was determined by performing multipoint linkage analysis. LOD scores for linkage (recombination fraction = 0) between PS deficiency and the four microsatellite markers are shown in Table 1. Four of the families showed very high LOD-scores between 3.2 and 4.8. Three of the remaining four families produced lower but positive LOD-scores between 0.78 and 1.2. The four families with high LOD-scores contained between 16 and 31 individuals, whereas the families with slightly lower LOD-scores contained between six and eight individuals. The lower LOD-scores in three of the families are a direct result of their small sizes and the low number of affected individuals. In addition, family number 26 was uninformative. Family number 20 most likely contains a recombination event, which contributes to the lower LOD-score observed in this family. Thus, the positive LOD-scores detected in all informative families support cosegregation between the markers flanking the PROS1 gene and the PS deficiency phenotype.

Simwalk2 suggested three recombinant haplotypes in total, one of which is the above-mentioned in family number 13. This recombination event has occurred between D3S1595 and D3S1552, which correspond to the two divisions within the family. In family number 17, recombination has occurred between D3S2388 and D3S1595 or between D3S1595 and D3S1552. In family number 20, Simwalk2 suggested a recombination between D3S1595 and D3S1552. According to the

Fig. 1. Pedigrees demonstrating cosegregation between PS deficiency and microsatellite haplotypes of the PROS1 gene region. Arrows indicate index cases, which have previously been sequenced with no causative mutations found. Filled symbols indicate diagnosed PS deficiency, and the capital letter N denotes healthy individuals, whereas empty symbols indicate unknown status. We have had access to blood samples of index cases, which have previously been sequenced with no causative mutations found. Filled symbols indicate diagnosed PS deficiency, and the capital letter N denotes healthy individuals, whereas empty symbols indicate unknown status. We have had access to blood samples of

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order in which the markers and the PROS1 gene appear, none of the recombination events separated the gene from its two flanking markers.

In families 15 and 31, null-alleles appeared for some markers in the disease-associated haplotypes. As null-alleles are usually the result of polymorphisms occurring in the primer annealing sites or, more seldom, the result of deletions, the marker D3S3634 was re-analyzed using a different set of primers. Also in these cases, the disease-associated haplotypes carried a null-allele in both families. This indicates that deletions exist in both of these families. This pattern of inheritance is illustrated in Fig. 1(C) where the hemizygosity for marker D3S3634 result in several cases of apparent non-Mendelian inheritance in family 31.

**Discussion**

The identification of PROS1 mutations in PS deficiency has been reported in several studies, including more than 300 unrelated PS deficient families [10,17,23–33]. In 53% of all these cases a mutation, likely to cause the decreased level of PS, was found, leaving the remaining 47% unexplained. Different screening procedures for mutation detection have been used in these studies including sequence analysis via RT-PCR, single-strand conformation polymorphism (SSCP) analysis, heteroduplex analysis, analysis by denaturing gradient gel electrophoresis (DGGE) and PCR amplification of PROS1 exons followed by direct sequence analysis. The mRNA expression is fairly rapid but mutation detection is limited by the occurrence of allelic exclusion [7,25,38]. SSCP and DGGE analysis have been successfully applied as mutation screening procedures in many genes. However, the sensitivity of these techniques is not 100%. Thus, the preferred way to screen for mutations is by direct sequencing of the PROS1 coding sequences, although it is more expensive than other screening techniques. The direct sequencing approach has been used in the majority of studies, including 147 families with PS deficiency [10,17,23,25,26,29,32,33]. Despite using this approach, 46% of the cases did not reveal any PROS1 mutations. The sequencing approach was also used for our PS families with similar results, i.e. no mutation could be found in eight out of 17 PS deficient families [33]. This situation thus implicates either the existence of other loci involved in PS deficiency, or the existence of common types of genetic defects in the PROS1 gene region that are not easily detected by sequencing of the coding regions.

The aim of the current study was to determine whether there was cosegregation between certain microsatellite haplotypes of the PROS1 gene region and the disease phenotype in families in whom earlier sequencing had not revealed any causative mutations in the PROS1 gene. We found PS deficiency to cosegregate with the PROS1 haplotypes in seven of the eight families lacking an identified mutation, one family remaining uninformative [33]. The four large families (families 8, 13, 15 and 17) showed very high LOD-scores clearly demonstrating linkage. The smaller families (families 11, 20, and 31) showed lower but positive LOD-scores. As we only test one candidate locus for cosegregation, the existence of positive LOD-scores in all informative families is an argument for the involvement of PROS1 in a majority of protein S deficiency cases. In addition, in families 15 and 31 the cosegregating disease haplotypes carried deletions. Whether these deletions are causally related to the protein S deficiency phenotype remains to be demonstrated. Taken together, these results clearly indicate that in the informative families, the mutations causing PS deficiency are located in or within close proximity of the PROS1 gene locus.

Our results are not in accordance with the results of two other studies, where it has been suggested that the molecular basis of type III PS deficiency is not a single defect in the PROS1 gene that is inherited in a Mendelian way [10,39]. However, in this context it is noteworthy that type III PS deficiency is difficult to diagnose accurately due to a considerable overlap in PS values between individuals with and without the genetic defect. In addition, the number and sizes of the previously investigated type III PS deficient families were smaller than the ones now investigated. Moreover, we believe that the use of a panel of four informative microsatellite markers increase the validity of the present study. Based on our results, we propose that PS deficiency is a single-gene disorder with Mendelian inheritance and close to complete penetrance. This most likely includes most or all PS deficiency cases for which no causative mutations have been identified despite sequencing of the entire coding region. One explanation for the absence of identified mutations in previous studies might be that sequencing of the coding regions fails to detect mutations in the promoter or in the introns. As the promoter region of PROS1 is largely uncharacterized and the introns are rather large, the screening for mutations in these regions is an extensive and uncertain task, which is even further complicated by the existence of the homologous PROS2 pseudo gene. Other possible causes of protein S deficiency, which are difficult to identify with a sequencing approach, are larger deletions or duplications covering the whole or part of the PROS1 gene. Given the results obtained in the present study for families 15 and 31, a dedicated screen for deletions in all exons of the gene seems warranted.

An implication of our finding is that a PS deficiency diagnosis can now be confirmed genetically, provided an entire family with several affected family members is analyzed. By using informative microsatellite markers, the inheritance of a cosegregating haplotype can be identified. As the penetrance of PS deficiency is close to complete, expressing a disease-associated haplotype is a very strong indication for having the disease. Considering the difficulty with which PS deficiency is diagnosed phenotypically, this genotypic approach will facilitate diagnosing familial PS deficiency.

In conclusion, our data contribute to a clearer understanding of the genetic background of inherited PS deficiency in terms of cosegregation between the disease and the PROS1 gene locus. This new knowledge will help unravel the molecular genetics involved in PS deficiency.
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