Normal Functional Protein S Activity Does Not Exclude Protein S Deficiency

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Key Words

Laboratory diagnosis · Protein S deficiency · Screening

Abstract

Protein S (PS) deficiency appears to increase the risk of venous thrombosis. PS deficiency is classified into three phenotypes using antigenic levels and functional activity. By definition, all three phenotypes of PS deficiency should result in low activated protein C cofactor activity. We compared the results of functional PS activity testing to free antigenic PS testing in order to determine if a normal functional PS activity assay result could eliminate the need for free antigenic PS testing. The sensitivity of the functional assay is 45.5% (95% confidence interval, CI, 36–55%), specificity 95.3% (95% CI 93–97%), negative predictive value 88.6% (95% CI 86–91%) with a positive predictive value of 68.5% (95% CI 57–79%). In conclusion, a normal functional PS activity result does not exclude free antigenic PS deficiency. Functional PS activity testing should not be used as a screening test to eliminate free antigenic PS testing for the laboratory diagnosis of PS deficiency.

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Introduction

Protein S (PS) is a glycoprotein of $M_r = 69,000$ produced predominantly in the liver by a mechanism involving vitamin-K-dependent γ -carboxylation [1]. The active

gene for PS (PROS1) is localized on the third chromosome. PROS1 spans over 80 kb and contains 15 exons interrupted by 14 introns [2]. PROS2, an inactive pseudogene, is found on the same chromosome [2]. PS circulates in plasma in free and bound forms. Thirty to 40% of PS circulates in the free form with the remainder bound to the C4b-binding protein [3]. Only free PS is biologically active (i.e. only free PS acts as a cofactor of activated protein C, APC, in the coagulation cascade). In the presence of calcium and phospholipid surfaces, PS binds to APC in a 1:1 complex and acts as a cofactor for the inactivation of factor Va and VIIIa by APC. PS also directly inhibits the prothrombinase and tenase activity in an APC-independent manner. Hence, PS acts as an anticoagulant protein and its deficiency predisposes to venous thromboembolism.

PS deficiency is relatively rare, being present in 0.03–0.13% of the general population [4]. Prospective cohort studies demonstrate that those with PS deficiency have a risk of venous thrombosis of 0.4% per year [5, 6]. Patients with PS deficiency also have an 8-fold increased risk of development of secondary deep vein thrombosis (surgery, trauma, immobilizations, pregnancy and postpartum period) [7].

Classically PS deficiency has been classified into type I deficiency (quantitative deficiency with low total PS antigen levels, low free PS antigen levels and low APC cofactor activity), type II deficiency (qualitative deficiency with normal total PS antigen levels and normal free PS antigen levels but low APC cofactor activity) and type III deficiency (normal total PS antigen levels but low free PS

antigen levels and low APC cofactor activity). Recently, however, a new proposed classification system suggests that PS deficiency be categorized as quantitative deficiency (old type I and type III deficiency) and qualitative deficiency (type II deficiency) [8]. In the literature, over 131 mutations have been identified within *PROS1*, most within families with type I and type III phenotype of PS deficiency. Only seven mutations have been identified in patients with type II PS deficiency [8, 9]. It has been demonstrated that different PS phenotypes can result from the same mutation, calling into question the utility of phenotypic classifications.

The clinical laboratory diagnosis of PS deficiency remains controversial, difficult and costly [8]. The ideal screening test for PS deficiency should accurately identify patients at increased risk of venous thromboembolic events. Quantitative and qualitative assays are available on the market that can measure total PS antigen levels, free PS antigen levels and PS APC cofactor activity (functional assays). The best predictor of risks of venous thromboembolic events in patients with suspected PS deficiency appears to be PS genotype and the best predictor of genotype, among widely available laboratory assays, appears to be free PS antigen levels [10, 11].

In theory, all types of PS deficiency should result in low functional PS activity (i.e. APC cofactor activity). We sought to compare functional PS activity testing to free antigenic PS testing in order to determine if a normal functional PS activity test could eliminate the need for free PS antigen testing.

Materials and Methods

Functional PS Activity and Free PS Antigen Testing

Our study population consists of consecutive patients (inpatients and outpatients) referred for PS testing at a tertiary care hospital over a 12-month period. Patients underwent both free PS antigen and functional PS activity testing. Free PS antigen levels were determined using the Asserachrom free PS ELISA assay (Diagnostics Stago, Asnières, France). The Asserachrom free PS kit is intended for the quantitative determination of free PS in plasma by a one-step sandwich-technique ELISA. The kit was used according to the instructions of the manufacturers without any modifications. The immobilization of the antigen and of the immunoconjugate consisted of a 1-hour incubation at room temperature (18–25°C). Absorbance was measured at 492 nm. The reference range used for the free antigenic PS test was 0.54–1.17 U/ml. Functional PS activity was determined using the Acticlot Protein S kit (American Diagnostica, Greenwich, Conn., USA). Acticlot Protein S is a functional APC cofactor activity assay intended for the quantitative determination of PS APC cofactor activity. In the assay, dilutions of patient plasma are mixed with PS-depleted plasma. A reagent

Table 1. Distribution of free PS antigen measurements and the functional PS APC cofactor activity in the study population

Functional PS assay	Free PS levels		
	low levels	normal levels	total
Abnormal function	50	23	73
Normal function	60	465	525
Total	110	488	598

that contains factor Xa, APC and phospholipids is added to the mixture. Following a 5-min incubation period, calcium chloride is added to initiate clot formation. The sample dilution buffer contains 0.2 M NaCl, 0.03 M HEPES and a sufficient quantity of Polybrene® to neutralize up to 1.2 U/ml heparin in a plasma sample. The kit was used according to the instructions of the manufacturer without any modifications with a reference range of 0.57–1.21 U/ml. In a retrospective subset of our study patients (n = 364), APC resistance results were obtained from the laboratory information system.

Data Analysis

Pearson’s correlation coefficient was calculated to measure for an association between functional PS activity and free PS antigenic levels. Two-by-two tables were constructed to compare functional PS activity testing to free PS antigenic levels. Sensitivity, specificity, positive and negative predictive values and 95% confidence intervals (CI) for each of these were calculated. Statistical analysis was performed with the SPSS system software. Subgroup analyses in patients with (1) abnormal international normalized ratio (INR) and (2) previous APC resistance testing were conducted to eliminate the possibility of bias secondary to oral anticoagulant therapy and APC resistance.

Results

598 patients had duplicate functional PS activity and free PS antigen testing. 12.8% of our study patients had abnormal functional PS activity testing and 18.4% of our study patients had abnormal free PS antigen assays. The Pearson’s correlation coefficient comparing activity and antigen levels was 0.6 (p < 0.0001). Of 110 patients with free PS antigen levels below the normal range, only 50 (45.5%) had concomitantly low functional PS test results. Functional PS testing has a sensitivity of 45.5% (95% CI 36–55%), a specificity of 95.3% (95% CI 93–97%), a negative predictive value of 88.6% (95% CI 86–91%) and a positive predictive value of 68.5% (95% CI 57–79%) in detecting abnormal free PS antigen levels (table 1). The likelihood ratio for a positive test is 9.6 (95% CI 6.2–15.1) and the likelihood ratio for a negative test is 0.57 (95% CI 0.48–0.68).

Table 2. Distribution of free PS antigen measurements and the functional PS APC cofactor activity in a subgroup of the study patients with normal INR

Functional PS assay	Free PS levels		
	low levels	normal levels	total
Abnormal function	28	19	47
Normal function	51	446	497
Total	79	465	544

Over 50% of patients with an INR >1.2 had a free antigenic PS level below the normal limit. Table 2 demonstrates that the diagnostic test characteristics were comparable when patients with abnormal INR are excluded. In this subgroup, functional PS testing has a sensitivity of 35.4% (95% CI 24.9–46.0%), a specificity of 95.9% (95% CI 94.1–97.7%), a negative predictive value of 89.7% (95% CI 87.0–92.4%) and a positive predictive value of 59.6% (95% CI 45.6–73.6%) in detecting abnormal free PS antigen levels.

In a subset of 364 study patients we were able to obtain APC resistance testing. Thirty-six (9.9%) of these patients had abnormal APC resistance. Of 14 patients with false-positive functional PS testing, 6 had APC resistance.

Discussion

In our study, we have shown that PS APC cofactor activity testing (i.e. functional testing) should not be used as a screening tool for the diagnosis of PS deficiency. Given that the three types of PS deficiency should result in low functional PS (i.e. low APC cofactor activity), a clinical laboratory strategy whereby only samples with abnormal functional PS testing are further studied with antigenic assays to further classify the nature of the deficiency appeared to make sense. However, our study has shown that functional PS testing only has a sensitivity of 45.5% and a negative predictive value of 89% for free antigenic PS deficiency. Put another way, over 50% of the patients with a free PS antigen deficiency had a normal functional PS test (many false-negative results). Hence, many patients with free antigenic PS deficiency (which has been confirmed to be the best surrogate of genotype which in turn is the best surrogate of clinical risk [10, 11]) would be missed with a screening strategy using functional PS APC testing first.

Many factors can influence PS levels including oral anticoagulants, pregnancy, oral contraceptives, hormone replacement therapy, acute thrombosis, liver disease, vitamin K deficiency, disseminated intravascular coagulation and nephrotic syndrome [12–14]. To determine whether our results could have been influenced by severe liver disease, disseminated intravascular coagulation, vitamin K deficiency and/or oral anticoagulant therapy, we did a subgroup analysis in patients with a normal INR and were unable to demonstrate a difference in the test characteristics of functional PS testing.

Limitations of our study include that we could not control for oral contraceptive therapy, hormone replacement therapy or pregnancy, but these states reduce both functional and antigenic PS activity hence a systematic bias is unlikely [14]. Resistance to APC has been previously shown to interfere with prothrombin-time-based functional assay of PS [15]. This interference leads to artificially low levels of PS activity and to misclassification of thrombophilic patients. This phenomenon explains some [43% (6/14) in our substudy] of the 23 false-positive functional PS tests but many false-positive tests remain unexplained [57% (8/14)]. Furthermore, treatment with heparin can increase PS activity and result in false-positive functional PS [16]. The functional PS assay used in our study contains a heparinase minimizing the risk that this influenced our findings. Other laboratory issues can also influence PS testing including temperature, dilution and incubation times [17]. However, all samples were handled by the same protocol so we do not believe these factors influenced our study findings. We also could not control for the influence of acute thrombosis or the nephrotic syndrome. Finally, it has been reported that systemic lupus erythematosus may increase functional PS activity and result in false-positive functional PS testing [18]. We did not collect these data, and patients with systemic lupus erythematosus may have been tested as part of this study. Despite all the potential confounders of our findings it is important to acknowledge the limited applicability of screening out influences of clinical confounders in a clinical laboratory, i.e. even if we are able to identify a complete list of factors leading to false-positive and false-negative functional PS testing, a strategy of functional PS testing first would be difficult to apply in most clinical and reference laboratories (clinical data not available or not provided).

We acknowledge that these results may be unique to this assay method. Further research is required in order to compare the different PS APC cofactor activity assays to the antigenic testing. Furthermore, guidelines are need-

ed for clinical PS deficiency testing. Investigations are needed to determine reference ranges in different populations and standardization of laboratory protocols for testing for PS deficiency. These guidelines will help the clinician to make an accurate diagnosis of PS deficiency.

In conclusion, a normal functional PS activity test result does not exclude free antigenic PS deficiency. The functional PS activity testing should not be used as a screening test to eliminate free antigenic PS testing for the prediction of PS deficiency.

Acknowledgments

M.A.R. is the recipient of the Maureen Andrew New Investigator Award from the Heart and Stroke Foundation of Canada. Many thanks to Michelle Willson and Christine Gagne-Rodger for assistance in manuscript preparation and revision. Many thanks to Mary Calvert and Marisa Freedman for technical assistance and support.

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