行政院國家科學委員會補助專題研究計畫成果報告

S蛋白缺乏症的基因研究(II)

計畫類別：■個別型計畫　　□整合型計畫

計畫編號：NSC 88－2314－B－002－067

執行期間：87 年 8 月 1 日至 89 年 4 月 30 日

計畫主持人：蔡 偉

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中 華 民 國 89 年 9 月 25 日

政院國家科學委員會專題研究計畫成果報告

S蛋白缺乏症的基因研究（二）

Genetic Study of Protein S Deficiency (II)

計畫編號：NSC 88-2314-B-002-067
執行期限：87年8月1日至89年4月1日
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中文摘要

先天性S蛋白（PS）缺乏症是中國人遺傳性血栓好發症的最常見的原因。本研究之目的是研究中國PS缺乏家族的基因缺陷。我們利用allele-specific primers進行聚合酵素連鎖反應，並單股DNA結構多樣型分析、直接DNA序列分析，研究PS基因所有轉譯區（包括intron-exon交界處）及基因啟動子的部分。迄今我們完成研究33個PS缺乏家族，在26個家族發現十一種不同的基因缺陷，全是點突變（point mutations），絕大數是從前未曾報告的。其中八種突變發生exons上，二種突變在introns以致影響splicing acception，另一種突變在啟動子（promoter）致使基因無法轉錄。其中Tyr519stop突變重複發生於十三個家族，是中國病人最常見的基因缺陷。由這些基因變化，我們揭開PS基因與蛋白缺乏表現型二者的關係。另外我們頭一個發現在PS啟動子上的基因突變，必將有助瞭解啟動子結構與轉錄功能。

關鍵詞：S蛋白缺乏症、基因缺陷

Abstract

Congenital protein S (PS) deficiency is the most common disorder of hereditary thrombophilia in Chinese. The purpose of this study is to detect the genetic defects of this disorder. We amplified the interested gene fragments through allele-specific primers and polymerase chain reaction (PCR). We then detected mutations through single strain conformation polymorphism (SSCP) and direct DNA sequencing.

Among 33 unrelated families with PS deficiency, eleven different point mutations were detected from 26 families. Eight mutations occurred in coding regions, two at intron-exon splicing junctions, and one at the promoter. Most important, the novel non-sense mutation Tyr519stop was recurrent in thirteen families. It was the most common genetic defect in Chinese PS deficient patients. Our study disclosed the relationship of gene defects and phenotypes in Chinese patients. The novel PS promoter mutation would help understand the structure and the transcription function of the gene promoter.

Keywords: protein S deficiency, genetic mutations

Introduction

PS is a vitamin K-dependent plasma anticoagulant. It is known as a cofactor of activated protein C, which can inhibit coagulation factor Va or VIIIa[1]. In human plasma, approximately 60% of the circulating PS bound to C4b-binding protein. The bound form of PS has no cofactor activity of activated protein C (APC) [1], but still has ability directly to inhibit factors Xa and VIII.

PS is encoded by a PS-α gene, which had been cloned in 1986[2] and was known to contain 15 exons. Its gene structure was declared in 1990[3]. In the same year, an inactive pseudogene PS-β gene, highly homologous (97%) to the active PS-α gene, was found[4,5]. It increased the complexity and difficulty in the molecular genetic studies.

Deficiency of PS is an established risk for venous thromboembolism[1]. Clinically, total and free PS antigen levels, and APC cofactor activity levels can be measured. According these plasma levels, PS deficiency can be classified into three phenotypes[6]. Patients who had reduced antigen levels (total and free) and activities were defined as a type I PS deficiency. Patients who had normal antigen levels (total and free) but reduced activities were defined as a type II PS deficiency. Patients who had normal total antigen levels but reduced free antigen levels and activities of PS were defined as a type III PS deficiency.

We had previously reported that PS deficiency was the most common cause of hereditary thrombophilia in Taiwan[7]. The prevalence rate of PS deficiency in Chinese is much higher than that in Caucasian (35% vs. 2%, in venous thrombophilic families). To study the genetic defects of PS deficient patients is important to understand the racial difference.

In the first-year study, we succeeded to amply the PS-α gene by PCR method. However, the direct DNA sequencing is not efficient to find the mutations. In this second-year study, we modified study methods and got fruitful results.

Materials and Methods

Patients

In the Thrombosis Center of the National Taiwan University Hospital, we collected 33 unrelated PS deficient families with venous thrombosis from 1990 to 1999. All patients were confirmed to have PS deficiency by repeated blood samplings. Patient who had at least one family member with the PS deficiency was said to have a hereditary deficient state. Twenty normal persons were studied together as normal controls.

Blood sampling and measuring PS levels

All tested plasma samples were prepared using plastic syringes and balanced citrate as anticoagulant. Storage was at −70 ℃ and procedures were completed within 4 weeks. Total PS and free PS antigenic levels were determined by an electroimmunoassay using commercial kits (Diagnostica Stago, Asnieres, France). Rabbit polyclonal antibodies to human PS contained in the agarose gel were used to capture the antigen. Polyethylene glycol 8000 was used to precipitate the C4b bound PS for the measuring of free form PS. Functional assay for APC cofactor activity was performed through using commercial kits (Diagnostica Stago).

DNA preparation, PCR amplification

Genomic DNA was extracted from peripheral white blood cells by a commercial extraction kit. For amplifying and reading each exon and its intron-exon boundaries, we designed over 40 pairs of oligonucleotides as PCR or sequencing primers[8]. Because exon 5 is only 105 base-pairs far from exon 6, we amplified these two exons together. Using the difference of bases between PS-α and PS-β genes, we created allele-specific primers for PCR reaction. Only PS-α gene can be amplified, except for exon 3, which sequence is identical in true gene and pseudogene.

Single strain conformation polymorphism (SSCP)

Four microliters of the PCR product was mixed with 6 microliters of denatured buffer (95% formamide, 0.05% xylene cyanol, 0.04% bromophenol blue), then was denatured at 95 ℃ for 5 minutes. The solution was then applied to a 12.5% thin polyacrylamide gel (GeneGel Excel Kit, Pharmacia). Electrophoresis was carried out at 15℃, 35 mA, 600 V for 60 to 90 minutes. The SSCP pattern in the gel was detected by silver staining (Pharmacia).

Direct sequencing

The asymmetric PCR produced single-stranded DNA as sequencing template, which was purified by homemade CL6B-sepharose spin columns. Sequencing reaction was done using nested primers, S^{35}-dATP or dCTP, and Sequenase kit (Pharmacia). The sequences were demonstrated by the non-

denatured polyacrylamide gel electrophoresis.

Results

Total 33 unrelated families with PS deficiency were studied. The genetic defects were identified from 26 families (detection rate 79%, 26/33). Total eleven different point mutations were found (Table 1). One mutation was at the promoter region. Two intronic mutations were at the splicing acceptor sites. The other eight mutations were at the coding region.

Table 1 Genetic Mutations of 26 families

	Nucleotide mutation	Amino acid exchange	Family
1	C(-56)T	promoter region	5
2	GAA→GCA	Glu 16 Ala	1
3	CGT→TGT	Arg 314 Cys	1
4	AGC→AGA	Ser 355 Arg	1
5	TGC→TAC	Cys 434 Tyr	1
6	TAT→TCT	Tyr 444 Ser	1
7	TCT→CCT	Ser 512 Pro	1
8	TAT→TAA	Tyr 519 stop	13
9	CGG→TGG	Arg 520 Trp	1
10	AG→AT at splicing acceptor site of intron I	mRNA splicing defect	1
11	AG→AT at splicing acceptor site of intron L	mRNA splicing defect	1

The nonsense mutation Tyr519stop was recurrent in 13 families. Another novel C(-56)T mutation at the promoter region was recurrent in five families. The other nine mutations appeared in single families. Majority of these mutations were never reported before[9].

Analyzing of the plasma protein levels of probands and their families, majority of these detectable mutations seemed responsive to type I or type III PS deficiency, except the intronic mutation at exon I, Cys434Tyr, and Arg520Trp were apparently responsive to type II PS deficiency.

We also found a mutation AAT to AAC at code Asn 457. This mutation is recurrent in two normal controls, but never found in PS deficient patients. This mutation neither changed the amino acid, nor caused the splice defect. It was a neutral polymorphism. There are three reported polymorphisms which common in Caucasians, that is, AAT/AAC at Asn-457, Ser460Pro and CCA/CCG at Pro-626. We noted that the first two were rare in Chinese, but the last one was common.

Discussion

In this study, we succeeded to amplify all exons of PS-α gene. Each PCR-amplified product was confirmed from PS-α gene. Some amplified fragments (exon 5, 6, 8, 13, and 15), which not read by the direct sequencing, were subcloned into a plasmid, pCR-II (TA cloning kit). All these subclones have been confirmed from PS-α gene.

In 5'-noncoding region, several hundred base-pair segment had very high C+G content (75~80%), which even failed to sequence by subcloning into pCR-II plasmid. Finally, we inserted the PCR fragments into M13mp19 phage, and successfully read its sequence from ssDNA template. Up date, no mutation reported at PS promoter[9]. We found that the mutation C(-56)T was recurrent in five unrelated families. All of them did not have other mutations in the coding region of PS-α gene. All probands exhibited the similar phenotype of PS deficiency. Family study also demonstrated the cosegregation. Its biological significance will be further clarified.

Two novel intronic mutations were found at the splicing junctions at intron I and L respectively. Their nucleotide substitutions were similar, that is, a G-to-T transversion at position -1 of the 3'(acceptor) splice site. The vertebrate consensus sequences of splicing acceptor sites show that the AG dinucleotides 100% present at position -2 and -1[10]. This G-to-T transversion at position -1 may result in mRNA splicing defects[11]. Two symptomatic patients with mutation at intron L had type I PS deficiency, nevertheless, the

proband with mutation at intron I had type II PS deficiency (PS total antigen 100%, free antigen 70%, function 2%). Our data suggested that a dysfunction PS molecule might appear in the latter plasma. The real mechanism was worth to study. The analysis of patient's mRNA and plasma protein was being investigated.

The mutation Glu16Ala was located at the Gla domain. From the study of other vitamin-K dependent protein, the Gla-16 residue is not exposed to outside solvent, but provides the coordination sites for calcium ions[12]. The patient with mutation Glu16Ala showed type I PS deficient phenotype, total antigen 64%, free antigen 35%, and function 35%. We predict that the change of Glu-16 may interfere with the folding of Gla domain.

The major mutations in our Chinese patient occurred in the last three exons or intron/exon junction, (69%, 18/26 families). The most interesting finding was the nonsense mutation Tyr519stop, which was recurrent in 13 families. It accounted half of total detectable mutations. It may be the founder effect, and easily found in thrombotic patients.

Two probands had doubly heterozygotic mutations. The first proband had total antigen 41%, free antigen 25%, and function 1%. His father and mother had PS levels 105%/ 84%/36% and 102%/40%/36% (total antigen / free antigen / function), respectively. Patient and his father had a same mutation Cys434Tyr. This mutation was apparently responsive for a type II PS deficiency. Unfortunately, no another mutation was detectable in this patient or his mother by our method.

The second proband had recurrent deep vein thrombosis as early as age of 12. He was identified to have both Tyr519 stop and Arg520Trp mutations. His PS levels still was detectable, that is, 30%/13%/3% (total antigen / free antigen / function). Because the Tyr519stop was a nonsense mutation, it suggested that Arg520Trp should be responsive a type II PS deficiency.

There was no mutation detectable in our seven PS deficient patients (21%, 7/33 families). Our missing rate was similar to other current reports. The probable causes were the limitation of SSCP-PCR, or the larger gene deletion, or genetic defects in related unknown gene, rather than PS gene.

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