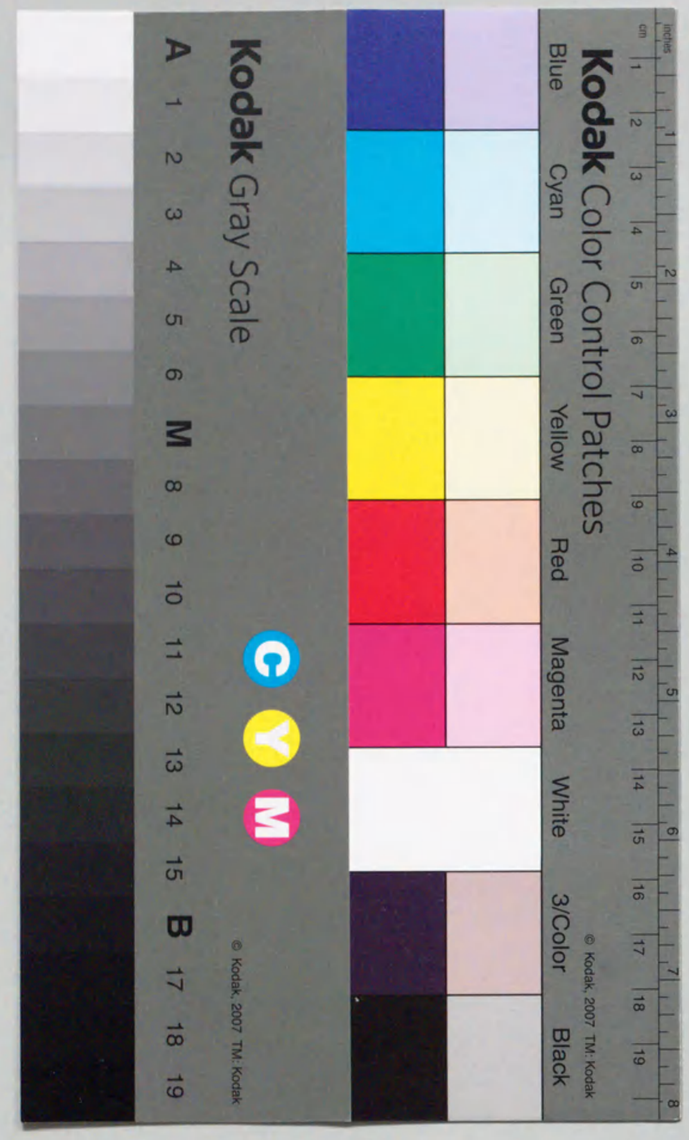

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HOMOZYGOUS PROTEIN C DEFICIENCY: IDENTIFICATION OF A NOVEL MISSENSE MUTATION THAT CAUSES IMPAIRED SECRETION OF THE MUTANT PROTEIN C

（先天性プロテインC欠乏症（ホモ接合体）：
蛋白の分泌障害を起こす一アミノ酸置換の同定）

山本　晃士

論　文　目　録

報告番号	※　第　　号	氏名	山本晃士

主論文

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The Journal of Laboratory and Clinical Medicine 掲載予定
Mosby-Year Book, Inc. 31枚

（既に印刷公表したものについては、その方法および年月日、未公表のものについては、公表の方法および時期を記入すること。）

副論文

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Genotype Establishments for Protein C Deficiency by Use of a DNA Polymorphism in the Gene.

（遺伝子多型性を利用した、先天性プロテインC欠乏症の遺伝子型の決定）

Blood, 77巻 12号 , 1991年 6月発行
The American Society of Hematology.

（　　同　　上　　）

参考論文

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Two novel sequence polymorphisms of the human protein C gene

（ヒトプロテインC遺伝子における２つの新しい遺伝子多型部位）

Nucleic Acids Research 19巻24号　1991年　発行
IRL Press at Oxford University Press

（　　同　　上　　）

先天性プロテインC欠乏症（ホモ接合体）：蛋白の分泌障害を起こす一アミノ酸置換の同定

Homozygous protein C deficiency: identification of a novel missense mutation that causes impaired secretion of the mutant protein C

Koji Yamamoto[1], Tadashi Matsushita[1], Isamu Sugiura[1], Junki Takamatsu[1], Eiichi Iwasaki[2], Hideo Wada[2], Katsumi Deguchi[2], Shigeru Shirakawa[2], and Hidehiko Saito[1,3]

山本晃士，松下正，杉浦勇，高松純樹，岩崎英一，和田英夫，土口克己，白川茂，斎藤英彦

[1]First Department of Internal Medicine,
Nagoya University School of Medicine
65 Tsuruma-cho, Showa-ku, Nagoya 466 JAPAN

[2]Second Department of Internal Medicine,
Mie University School of Medicine
2-174 Edobashi, Tsu, Mie 514 JAPAN

[3]Aichi Blood Disease Research Foundation
142 Aza-Machikita, Oaza-Moriyama, Moriyama-ku, Nagoya 463 JAPAN

Running Head: A new missense mutation in the protein C gene

This work is aided in part by grant No. 63637003 for scientific research on priority areas from the Ministry of

Education, Science and Culture and grants from the Ministry of Health and Welfare of Japan.

Correspondence: Dr. Hidehiko Saito
First Department of Internal Medicine,
Nagoya University School of Medicine
65 Tsuruma-cho,
Showa-ku, Nagoya 466 JAPAN
Phone: 81-52-741-2111 Ext. 2200
FAX: 81-52-741-1612

Reprint requests: Dr. Koji Yamamoto
First Department of Internal Medicine,
Nagoya University School of Medicine
65 Tsuruma-cho,
Showa-ku, Nagoya 466 JAPAN
Phone: 81-52-741-2111 Ext. 2203
FAX: 81-52-741-1612

ABSTRACT

We analyzed the promoter region, all the coding exons and exon-intron boundaries of the protein C gene of a Japanese patient with recurrent thromboembolism and complete protein C deficiency. By sequencing these fragments we identified a previously undescribed mutation. A guanine residue was replaced by an adenine residue converting Gly-292 (GGC) to Ser (AGC) in the last exon coding for the catalytic domain. Substitution of this key amino acid, invariably conserved in the serine protease superfamily to which protein C belongs, probably leads to destabilization of the tertiary structure. In a transient expression assay using COS 7 cells, the protein C level was extremely low in the culture medium of the cells transfected with the mutated protein C expression vector, as compared with that in the culture medium of the cells transfected with the normal vector. In contrast, small amounts of protein C was present in the cell extracts of the cells transfected with the mutated protein C expression vector, suggesting impaired secretion of the mutant protein C. Using mutagenic primers to introduce a new *Pvu*II site into the mutant allele, we made a study of the family members in this patient's pedigree, revealing that the mutant allele had been inherited in the affected individuals in this pedigree.

INTRODUCTION

Protein C, a vitamin K dependent plasma glycoprotein of 62,000 molecular weight, is a zymogen of a serine protease (1, 2). Once activated, protein C plays a key role in the "protein C anticoagulation pathway" in which it is responsible for proteolytic inactivation of coagulation cofactors Va and VIIIa (3). It also stimulates fibrinolysis by reducing the activity of tissue type plasminogen activator inhibitor (PAI) (4).

The physiological importance of protein C is clear from the fact that congenital protein C deficiency predisposes the individual to the development of thromboembolic diseases (5, 6). Most patients who are protein C deficient are only partially deficient and have approximately 50% of the normal level of protein C activity (7). Clinical symptoms found in deficient individuals include deep vein thrombosis, pulmonary embolism and cerebral infarction. Some partially deficient individuals have relatively infrequent thromboembolic episodes, which has led to the claim that partial protein C deficiency alone should not be characterized as an important risk factor (8).

Individuals who are completely deficient in protein C or have extremely low levels of plasma protein C are sometimes identified as having purpura fulminans as newborns (9, 10).

However, some of these individuals survive beyond the neonatal period into adulthood with or without histories of thrombosis (11). Thus, the spectrum of clinical manifestations caused by protein C deficiency is rather wide. Usually protein C deficiency is thought to be transmitted as an autosomal dominant with a high degree of penetrance, but in families with completely deficient individuals, the mode of inheritance has been classified as an autosomal recessive.

Since the protein C gene has been cloned (12) it has become feasible to study hereditary protein C deficiency at the molecular level. It is possible that the phenotypic heterogeneity of this disorder may reflect underlying heterogeneity at the molecular level. Thus studying DNA sequence abnormalities may be helpful in explaining the broad range of clinical severity of protein C deficiency. Only a handful of cases, however, have so far been analyzed at the DNA level (13-16).

We sequenced the promoter region, all of the coding regions and splice junctions of the protein C gene in a young woman with complete protein C deficiency. We identified a novel point mutation located in exon IX which would cause substitution of a serine in place of a conserved glycine residue at amino acid position number 292. Furthermore, we have shown that this point mutation was

inherited by the affected individuals in this family. To elucidate the mechanism by which the missense mutation causes protein C deficiency we have performed a transient expression assay using COS 7 cells. The mutant protein C was secreted into the culture medium at a very low level, indicating that this mutation is the molecular basis for protein C deficiency in this pedigree.

METHODS

Description of the patient and her family

The patient is a 31-year-old woman who reached adulthood without clinical evidence of thrombosis. Her first thromboembolic episode occurred at the age of 31 and was an infarction in the right lateral cerebrum. Since then, she has had recurrent episodes of venous thrombosis in both of her legs as well as mesenteric venous thrombosis. The protein C activity and antigen levels in this patient were undetectable (less than 5% of normal), while the activities of antithrombin III, protein S and plasminogen were within the range of normal. Her mother died at 35 years of age due to cerebral thromboembolism and her brother died at birth for unknown reasons. Figure 1 is a pedigree of her family and shows that protein C activity levels of her father and

other relatives are low. It also indicates that her parents were first cousins. From the pedigree alone it appears that the patient is homozygous and that her family members are heterozygous for protein C deficiency.

Plasma and DNA samples

Blood samples were drawn from the patient, her family members and normal individuals after informed consent was obtained. Nine parts of blood were mixed with one part of 3.8% trisodium citrate and the plasma was separated by centrifugation at 34000 g for 15 min at 4°C, then stored at -80°C until use. Genomic DNA was isolated from peripheral blood leukocytes by phenol extraction as previously described (17).

Protein C and other assays

Protein C anticoagulation activity was determined by using protein C-deficient plasma as a substrate and Protac^{R} (Boehringer Mannheim, Mannheim, Germany) as an activator of protein C (18) (STACLOT PROTEIN C^{R}, Boehringer Mannheim). The protein C antigen level was measured using a polyclonal rabbit serum against human protein C (ASSERACHROM PROTEIN C^{R}, Boehringer Mannheim).

Protein C activity and antigen levels were expressed as percentages of that observed in pooled plasma collected from

20 normal individuals. The pooled plasma was arbitrarily defined as having 100% protein C activity and antigen levels. Assays for the activities of antithrombin III, protein S, and plasminogen were performed as described earlier (19).

Preparation of oligonucleotide primers

We designed eight pairs of oligonucleotide primers for PCR (shown in Table 1) which covered the 5' untranslated (presumed promoter) region and all nine exons and exon-intron boundaries of the protein C gene. These primers have an *Eco*RI restriction endonuclease cleavage site which is convenient for subsequent cloning and sequencing. All primers were prepared as described earlier (20).

Polymerase chain reaction (PCR)

PCR was performed as previously described (21) with the following minor modifications (22). A 100 µl aliquot of the PCR reaction mixture contained 1 µg of genomic DNA in 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 200 µM of each dNTP, 100 pmole of each primer and 2.5 U of recombinant Taq DNA polymerase (Perkin Elmer-Cetus, Norwalk, CT). The mixture was overlaid with mineral oil to prevent condensation and subjected to the following series of steps for a total of 35 amplification cycles. The

samples were denatured at 94°C for 1 min, annealed at 60°C for 2 min, and heated to 72°C for 2 min for polymerization. The reactions were performed in an automatic thermal cycler (Perkin Elmer-Cetus).

DNA sequencing

The PCR products were digested with restriction endonucleases then subcloned into M13 vectors. These subcloned PCR products were then sequenced by the dideoxy chain termination method as described previously (20, 23).

Constructions of expression vectors

An expression vector for the normal protein C (pEUK-PC) was constructed by inserting a cDNA fragment coding for protein C into the pEUK-C1 vector (Clontech, Palo Alto, CA) as follows:

The cDNA coding for wild-type protein C was provided by Dr. Earl Davie (Seattle, WA) in pUK19. Between two *Eco*RI sites, the cDNA for protein C contained 69 noncoding bases 5' of the ATG initiation site, 126 bases of leader sequence, 1383 bases coding for the entire protein C molecule, and 202 noncoding bases 3' of the TAG stop codon, this latter region including two poly A signals. This fragment was excised from the pUK19 vector by *Eco*RI digestion and then ligated into the multiple cloning site of the pBluescript KS II(+)

vector. The insert direction was confirmed by digestion with *Nae*I. For insertion into the mammalian cell expression vector, the cDNA was excised from the pBluescript KS II(+) by employing an *Xba*I-*Xho*I restriction digestion and inserted into these same restriction sites of the vector pEUK-C1 which has the SV40 late promoter 5' upstream of the *Xba*I site.

Mutagenesis

Site-directed mutagenesis of the protein C coding sequences was performed using an Oligonucleotide-directed in vitro mutagenesis system^{R}, version 2.0 (Amersham, Arlington, IL) (24,25). We prepared the mutant oligonucleotide:

5'-AGGGTCTCCTGGCTGGCCTGATTGAGCTCG-3'

with a DNA synthesizer, Model 391 from Applied Biosystems (Foster City, CA). The mutant protein C cDNA was sequenced by dideoxy chain termination technique as described earlier (20), using the double-strand DNA as template, to verify the mutation and the fidelity of the remaining sequence. The fragment containing the mutated protein C was inserted into the expression vector (pEUK-mPC).

Transient expression of recombinant protein C

COS 7 cells were grown in Iscove's modified Eagle's medium containing 10% fetal bovine serum and 500 ng/ml of vitamin K

in 35 mm wells. Cells ($3x10^5$ per well) were transfected with the pEUK-C1 vector containing either the normal or mutant protein C cDNA by the DEAE-dextran technique (26). The culture medium was removed at 48 hours after the transfection for protein C measurement. The cells were lyzed with 0.1% NONIDET P-40 solution containing 10mM phenylmethylsulfonylfluoride after washing with PBS and the cell extracts were collected.

RESULTS

Analysis of the protein C gene

M13 phage clones, each containing one of the nine exons of the protein C gene originating from an individual who is homozygous for protein C deficiency, were sequenced by the standard dideoxy method. These sequences were compared to the normal protein C sequence. Only a single nucleotide change was found in all 9 exons of this protein C gene. A transversion in the last exon of the gene from guanine to adenine (GGC to AGC) converted Gly-292 to Ser (Fig. 2). This mutation was confirmed by sequencing five independent clones from this region.

Analysis of the family members

To determine whether this mutation was hereditary, we examined the DNA from family members of this patient using PCR amplification with mutagenic primers (20,27,28) as follows:

5'-CTCAGGAATTCGCCACTGGGGAGAGGCTCC-3'

5'-AGCCCCAGCCCGTCACGAGGGTCTCCC*A*GC-3'

After PCR amplification of the segment of the protein C genomic sequence in which this mutation occurred, the products were digested with *Pvu*II (CAGCTG), as shown in Figure 3. The mutant alleles contain this restriction site and their digestion yielded two fragments of 271 and 29 bp in length. The normal allele did not have this restriction site, so *Pvu*II digestion of that allele yielded a single 300 bp fragment. Thus family members carrying the mutation were identified as those whose DNA was shown by this analysis to contain two restriction fragments of 271 and 29 bp in length.

When the genomic DNA fragments of Japanese individuals with normal protein C activity and antigen levels were subjected to this analysis, *Pvu*II digestion yielded only a single 300 bp fragment in all cases, indicating that these individuals are all homozygous for the normal allele. *Pvu*II digestion of PCR products from the patient's family members revealed that only DNA isolated from her paternal grandfather (I-1) and paternal uncle (II-1), who both had

normal protein C activity and antigen levels, did not yield the two restriction fragments which indicate the homozygosity for the normal allele (Fig. 4). Clones obtained from the family members who are partially deficient in protein C (II-2, II-3, II-4 and II-5), produced two bands after *Pvu*II digestion: a 300 bp fragment and a 271 bp fragment, indicating that they each carry 1 mutant allele and 1 normal allele. (The 29 bp fragment could not be detected in these gels.) DNA taken from the proband (III-1), who is completely deficient in protein C, had only the 271 bp fragment after *Pvu*II digestion, confirming that she is homozygous for the mutant allele.

Transient expression of recombinant protein C

To ensure that the missense mutation identified in this pedigree could cause the deficiency of protein C, we constructed a protein C expression vector, pEUK-mPC, containing the abnormal sequence, as described in Methods, and transfected it into COS 7 cells for a transient expression assay. An analysis for protein C in the culture media and in the cell extracts, as assayed by ELISA using a rabbit polyclonal antibody, is shown in Table 2. About 90% of the normal recombinant protein C synthesized in the COS 7 cells was secreted into the culture medium, whereas the level of the mutant protein C secreted was only about 2% of

that of the normal recombinant protein secreted. Their cellular contents of protein C, however, did not differ significantly, suggesting impaired secretion of the mutant protein C.

DISCUSSION

The molecular basis of hereditary protein C deficiency is almost completely unknown in the majority of patients afflicted with this problem. Romeo *et al.* have reported that mutation of a single nucleotide (a C to T transition) at Arg-306 in one pedigree of a heterozygous deficiency results in a nonsense mutation. They also described a mutation in which a G to C transversion caused substitution of Cys for Trp-402, a missense mutation (13). In the latter case, the disulfide bonds generated by the variant cystein residue are thought to interfere with the correct folding of the protein, resulting in destabilization of the molecule.

Protein C Tochigi (14) and protein C London (15) have also been reported to be abnormal protein C molecules. In both cases, a C to T transition causing replacement of Arg-169 with a tryptophan residue, at the site of protein C activation by the thrombin-thrombomodulin complex, is thought to be responsible for defective activation of the

molecule and to result in thromboembolic disorder. More recently, a preliminary report by Gandrille *et al.* (16) described the identification of two mutations in exon VI (corresponding to exon VII in the report of Plutzky *et al.*, 29) which encodes the linker and activator portions of the protein C molecule. One of these was a C to T transition which created a nonsense codon at Arg-157 and the other was a G to A transition converting Arg-178 to Gln. Further details of the effect of these mutations on functional aspects of protein C have not been described.

We have identified a novel point mutation in the protein C gene of an individual who is completely deficient in protein C, which causes Gly-292 to be replaced by Ser (Fig. 2). We sequenced the entire protein C gene in this patient, and found only this single point mutation, therefore it is thought to underlie the clinical manifestations of protein C deficiency in this patient. To our knowledge, this is the first report describing the DNA abnormality in an individual with apparent homozygous protein C deficiency.

Glycine-292 structurally corresponds to glycine-133 in eukaryotic serine proteases and in bacterial trypsin (30). This residue is invariably conserved in serine proteases which contain this biochemical domain, including bovine prothrombin, the trypsinogens, the chymotrypsinogens and pig kallkrein. Computer simulation of the three-dimensional

structure of trypsin indicates that it has one α-helix, two six-stranded β-sheets, and six disulfide bonds (31). Glycine-133 of trypsin belongs to the group of residues which form β-sheets and is located at the base of the molecule (31). It is inferred that replacing a nonpolar glycine residue with a polar serine residue at this site in the protein C molecule influences the three-dimensional structure, leading to its destabilization or perhaps inactivation.

Our transfection experiment data appear to provide strong evidence for the impairment of the secretary process of the mutant protein C, since this mutation significantly reduced the amount of the mutant protein C secreted into the medium, while the cellular content was almost same with normal recombinant protein C (Table 2). There is much evidence that abnormal secretary or plasma membrane proteins are often specifically retained within the rough endoplasmic reticulum (RER) and are not secreted (32). Conceivably, this missense mutation causes significant alteration of protein C which in turn probably causes a substantial change in its secondary and tertiary structure, thereby affecting its posttranslational transport through the intracellular secretory compartments. The *in vitro* study also suggests the instability of the mutant protein C within the cells, possibly due to the altered conformation, because no

abnormal intracellular accumulation of protein C was observed in spite of the apparently retarded secretion.

The analysis we describe using PCR with mutagenic primers (Fig. 3) followed by *Pvu*II digestion provides a relatively easy screening procedure for this protein C mutation. The variant restriction pattern we describe here correlates completely with protein C deficiency in this pedigree and is absent in all the individuals we have examined which have normal protein C activity and antigen levels. The proband apparently inherited two copies of the same mutation, one from each of her parents, who were cousins. It also appears that the 50% reduction of protein C levels found in several individuals in this pedigree can be attributed to this missense mutation, since all of those individuals are heterozygous for the mutation.

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FIGURE LEGENDS

Figure 1 The pedigree of protein C deficiency in the family of an individual who is completely deficient in protein C. The proband is indicated by an arrow. Heterozygous individuals are indicated by a dot in the middle of the circle (females) or square (males). Protein C enzyme activity and antigen levels are indicated for each individual in the pedigree and are represented as the percentage of the amount of activity or antigen found in pooled plasma from 20 normal individuals. Normal protein C activity is considered to fall into the range of 78.3 to 121.7% of the protein C activity found in the pooled blood. Enzyme activity is written above the bar and antigen level is shown below the bar.

Figure 2 A novel missense mutation in the protein C gene isolated from an individual who is completely deficient in protein C. The nucleotide sequence shown is from the coding strand near the 3' end of exon IX of the protein C gene isolated from the patient's DNA. The nucleotide sequence is indicated along with the corresponding amino acids and residue numbers. A guanine nucleotide is replaced by an adenine in amino acid number 292, resulting in the

conversion of a glycine to a serine residue in the last exon of the mutant allele.

Figure 3 Strategy for direct detection of a single nucleotide substitution using mutagenic primers. The mutation, substitution of a guanine residue with an adenine residue in exon IX of the protein C gene, is indicated by white letters. We used mutagenic primers to replace a C residue (nucleotide 8592) with a T residue (indicated by T*) and an A residue (nucleotide 8593) with a G residue (indicated by G*). This created a new *Pvu*II site (CAGCTG) in the fragments produced by amplification of mutant alleles, and yielded two fragments of 271 bp and 29 bp in length after enzyme digestion. After amplification, the normal allele does not have the restriction enzyme cleavage site and generates only a 300 bp fragment after *Pvu*II digestion.

Figure 4 Analysis by PCR amplification followed by *Pvu*II digestion of the protein C alleles in the family members of an individual who is homozygous for protein C deficiency. A region of exon IX in the protein C gene was amplified by PCR using the mutagenic primers described in Figure 3 and shown in Table 1. After amplification by PCR, the exon IX fragments were digested with *Pvu*II and subjected to PAGE in

a 9% gel, then stained with ethidium bromide. We used DNA Molecular Weight Marker 4(ϕX174/HaeIII digest)^{R} from Nippon gene, JAPAN as the marker on the right side. The 29 bp fragment was not detected in this gel. However, the gel shows that *Pvu*II digestion of exon IX from all of the individuals in the pedigree who were partially deficient in protein C, produced bands of 271 bp and 300 bp in length. The 271 bp fragment results from digestion of the mutant allele and the 300 bp band results from digestion of the normal allele. DNA from exon IX of protein C from the individuals in the pedigree who are not deficient for protein C by enzymatic and antigenic analysis (I-1 and II-1), yielded only a single band of 300 bp in length after *Pvu*II digestion.

Abbreviations: DNA = deoxyribonucleic acid

PCR = polymerase chain reaction

Tris = Tris (hydroxymethyl) aminomethan

dNTP = deoxyadenosine, deoxyguanosine, deoxycytidine, or deoxythymidine triphosphate

Arg = arginine

Trp = tryptophan

Cys = cystein

Gln = glutamine

Gly = glycine

Ser = serine

RER = rough endoplasmic reticulum

TABLE 1 Oligonucleotide Primers and Amplified Protein C Gene Fragments

Primer	Nucleotide positions * 5'----------3'	PCR Products (bp)	Region
A-1	AGGCAGAATTCGGCTTCGGGCAGAACAAGC (-1641 to -1612)	206	Exon I
A-2	TCATAGAATTCCCTGGAGGGGGACTCACAG (-1449 to -1420)		
B-1	ACCCTGAATTCCAGCTTCCGCCTGACGGCC (-149 to -110)	309	Exon II
B-2	ACCCAGAATTCAGAGAGATGGTGGAAGCTG (146 to 175)		
C-1	CCTCAGAATTCCTCATGGCCCCAGCCCCTC (1260 to 1289)	262	Exon III
C-2	CCCTGGAATTCATCCTCTGGACCCATGGTG (1508 to 1537)		
D-1	TGCAGGAATTCGAGCCTGCCCGCTCTCTCC (2928 to 2957)	548	Exon IV,V,VI
D-2	AGCGTGAATTCTGGGCGATGTATTGGGGGC (3462 to 3491)		
E-1	GGGAGGAATTCCTGGCAGGCCCCTCACCAC (6078 to 6107)	232	Exon VII
E-2	AGGATGAATTCAGTGATCCCGGACCCAGCA (6297 to 6326)		
F-1	GACTGGAATTCGTCAGGAGGCAGCCCTGTG (7084 to 7113)	386	Exon VIII
F-2	TGCCCGAATTCGAAGAGGGCACCAGAGAAC (7457 to 7486)		
G-1	CTCAGGAATTCGCCACTGGGGAGAGGCTCC (8321 to 8350)	373	Exon IX
G-2	CCACGGAATTCTTGATGAAGTTGAGGACGA (8681 to 8710)		
H-1	CTCGTGAATTCCTGGGGCTACCACAGCAGC (8600 to 8629)	445	Exon IX
H-2	AGAACAGAATTCCGGTGTGCTTGTTACATG (9032 to 9061)		

* Primer sequences and numbers are according to the nucleotide sequence of Foster et al. (12) except for the underlined EcoRI restriction sites.

Table 2. Expression and Secretion of Normal and Mutated Recombinant Protein C from COS cells*

Plasmid	PC in media (ng)	PC in cell extract (ng)	PC secreted (%)
none	0	0	0
pEUK-PC	63.0	6.03	91
pEUK-mPC	1.18	8.84	12

*Culture media were collected from 35-mm plates 48 h after transfection with protein C expression plasmids. The cells were harvested with trypsin, washed twice with PBS, resuspended in PBS, and lysed by 0.1% NP-40 solution. Protein C levels in the culture media and in the cell extracts were measured by ELISA. The result was a mean of five separate experiments.

PC : Ac
PC : Ag

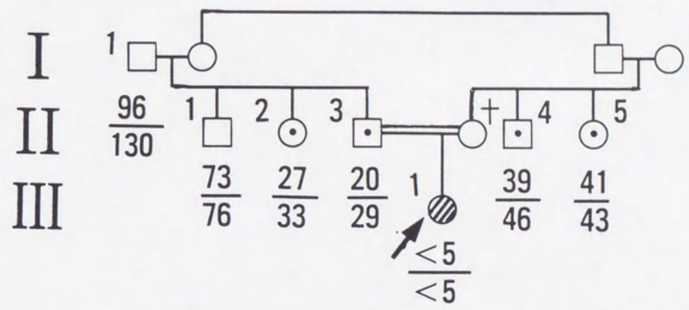


Fig. 1

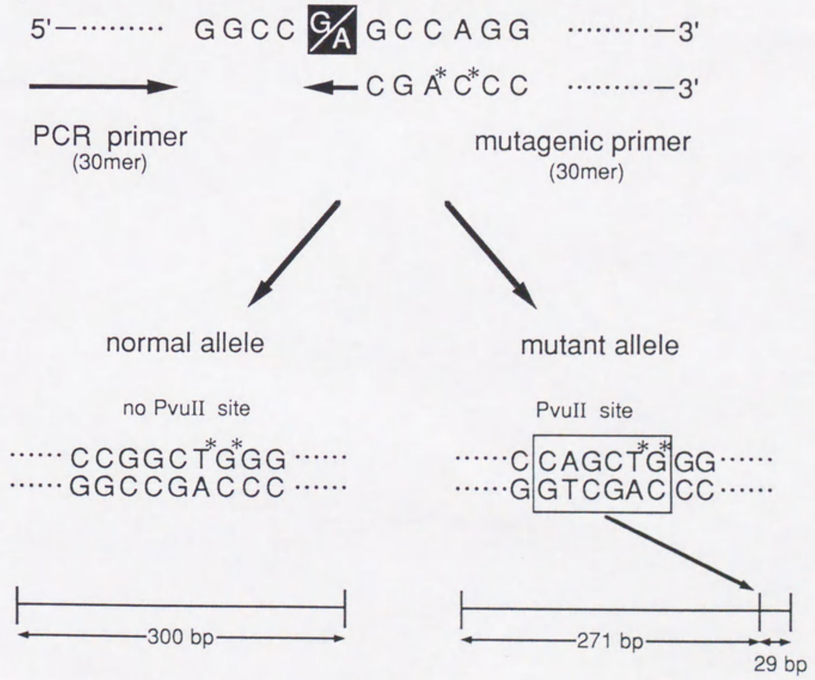
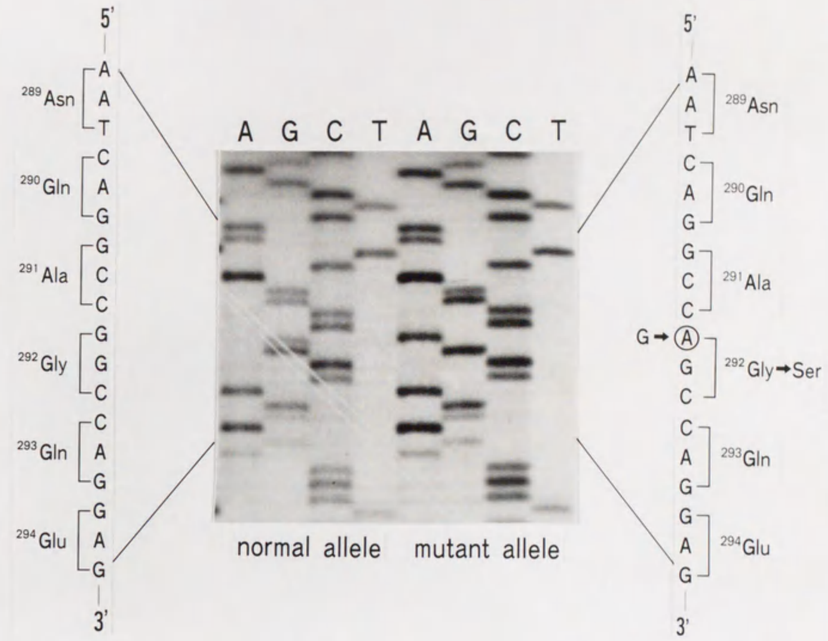


Fig. 3


5'
A
A
T
289 Asn
C
A
G
290 Gln
G
C
C
291 Ala
G
G
C
292 Gly
C
A
G
293 Gln
G
A
G
294 Glu
3'
A G C T A G C T
normal allele
mutant allele
5'
A
A
T
289 Asn
C
A
G
290 Gln
G
C
C
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C
292 Gly → Ser
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A
G
293 Gln
G
A
G
294 Glu
3'

Fig. 2

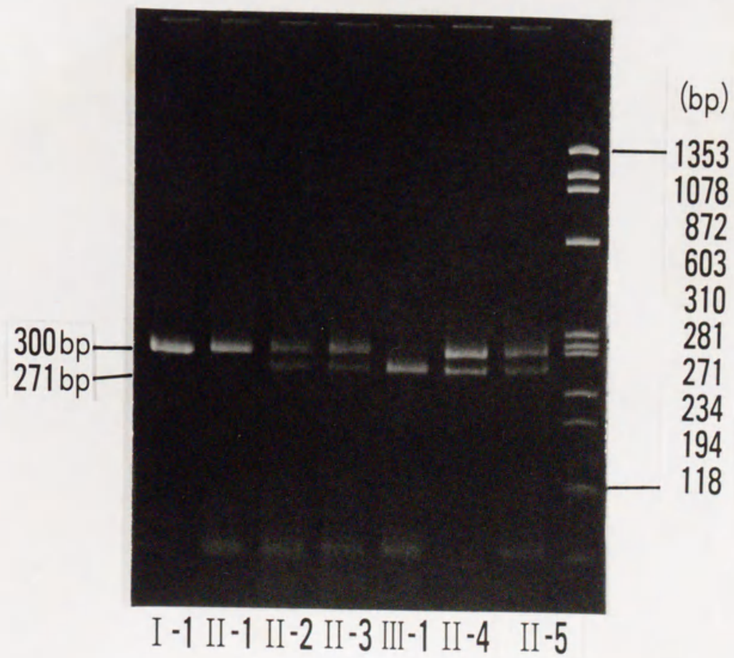
300 bp
271 bp
(bp)
1353
1078
872
603
310
281
271
234
194
118
I-1 II-1 II-2 II-3 III-1 II-4 II-5

Fig. 4

副　論　文

「遺伝子多型性を利用した、先天性プロテインC欠乏症の遺伝子型の決定」

Genotype Establishments for Protein C Deficiency by Use of a DNA Polymorphism in the Gene

By Koji Yamamoto, Mitsune Tanimoto, Tadashi Matsushita, Kazuo Kagami, Isamu Sugiura, Motohiro Hamaguchi, Junki Takamatsu, and Hidehiko Saito

山本晃士，谷本光音，松下正，利見和夫，杉浦勇，浜口元洋，高松純樹，斉藤英彦

During the course of structural gene analyses for protein C deficiency, we have confirmed that a T or G nucleotide variation is present at exon 6 of the protein C gene. This single-base substitution was located at the third nucleotide coding for Ser (TCT) at 99 residue, and neither produces an amino acid substitution nor creates a new restriction enzyme site. By using mutagenic primers that could introduce A instead of G at the third nucleotide 3' to the de novo polymorphic site, we have created the polymorphic *Xba* I site (T/CTAGA) in a more-frequent allele. Polymerase chain reaction using these mutagenic primers and subsequent *Xba* I digestion of 20 normal Japanese genomic samples showed that the frequency of this new sequence polymorphism designated as PC-493 was 0.18 and that the estimated heterozygosity rate was 28.9%. In Caucasians, the frequency of this polymorphism was 0.25, and a significant difference did not exist between Japanese and Caucasian populations. The examination of the haplotype inter-relationships with PC-493 and the *Msp* I polymorphism 5' to the protein C gene established that PC-493 gave a 16.7% chance of new information per individual for people who were previously homozygous for the *Msp* I polymorphism. We have performed a family study of the protein C-deficient pedigree using this sequence polymorphism, and found that the PC-493 DNA polymorphism was a useful marker for tracing the affected gene in protein C-deficient family members.

PROTEIN C IS THE vitamin K-dependent plasma zymogen of an antithrombotic serine-protease, activated protein C.[1,2] Protein C is an important regulator of blood coagulation and a congenital deficiency of this plasma protein is often associated with a high incidence of thromboembolic episodes.[3-6] Protein C deficiency has been classified as an autosomal dominant disease with a high degree of penetrance, but in certain families with homozygous patients the mode of inheritance of protein C deficiency has been classified as autosomal recessive.[7]

In this report, we have confirmed that a previously noted single nucleotide variation (a T or G) in exon 6 of the protein C gene[8-11] is present among the normal Japanese population. Polymerase chain reaction (PCR) amplification with mutagenic primers and subsequent digestion with *Xba* I endonuclease were found to be useful for detecting this single-base substitution. By using this strategy we have successfully performed family diagnosis in a pedigree of protein C deficiency.

MATERIALS AND METHODS

Patients and DNA samples. Venous blood samples were collected in a 1/10 vol of 3.2% (wt/vol) trisodium citrate from normal individuals, patients with heterozygous protein C deficiency, and their family members after informed consent was obtained. Genomic DNA was isolated from peripheral blood leukocytes by phenol extraction as previously described.[12] The patient (M.U.) was a 46-year-old man who had recurrent episodes of venous thrombosis, pulmonary embolism, and cerebral infarction. He had 42% protein C activity. A family study was performed in the pedigree of a 2-year-old girl (Y.T.) who suffered from venous thrombosis in her right leg. Her protein C activity was 32%.

Protein C assays. Protein C amidolytic activity was determined by using protein C-deficient plasma as a substrate and Protac as an activator for protein C[13] (STACLOT PROTEIN C, Boehringer Mannheim, Germany). Protein C antigen was measured by using ASSERACHROM PROTEIN C (Boehringer Mannheim). The activity and antigen of protein C were expressed as the percent of the normal pooled plasma. The normal pooled plasma from 20 normal volunteers was arbitrarily defined to contain 100% activity and antigen. We have now given a normal range (100% ± 21.7%) for the protein C activity assays.

Preparation of total sets of oligonucleotide primers. We designed eight pairs of oligonucleotide PCR primers covering 5' untranslated presumptive promoter region and all eight exons and intron-exon boundaries of the protein C gene. These primers have suitable restriction endonuclease sites convenient for subsequent cloning and sequencing. All of the primers were prepared on a DNA synthesizer from Applied Biosystems (Foster City, CA) and purified with an oligonucleotide purification cartridge (Applied Biosystems) according to the manufacturer's recommendation.

PCR. PCR was performed as previously described by Saiki et al[14] with the following minor modifications. Fifty microliters of PCR reaction mixture contained 0.25 μg of genomic DNA in 67 mmol/L Tris-HCl (pH 8.8), 6.7 mmol/L $MgCl_2$, 16 mmol/L ammonium sulfate, 1 mmol/L 2-mercapto-ethanol, 10% dimethyl sulfoxide, 1.5 mmol/L of each deoxyribonucleotide, 50 pmol of each primer, and 2.5 U of recombinant Taq DNA polymerase (Perkin Elmer-Cetus, Norwalk, CT). The mixture was overlaid with mineral oil to avoid condensation and subjected to the following total 35 cycles of amplification. The samples were denatured at 94°C for 1 minute, annealed at 63°C for 1 minute, and subsequently heated to polymerize at 72°C for 1.5 minutes. The reaction was performed with an automatic thermal cycler (Perkin Elmer-Cetus).

DNA sequencing. The PCR products were digested with restriction endonucleases and then subjected to 9% polyacrylamide gel electrophoresis. Each digested subfragment was recovered by electroelution (2014 Extraphor; Pharmacia-LKB Biotechnology, Uppsala, Sweden) and ligated into M13 multi-cloning sites with T4

From the First Department of Internal Medicine, Nagoya University School of Medicine, Nagoya, Japan; and the Aichi Blood Disease Research Foundation, Aichi, Japan.

Submitted August 13, 1990; accepted February 5, 1991.

Supported by Grant No. 63637003 for Scientific Research on Priority Areas from the Ministry of Education, Science and Culture in Japan.

Address reprint requests to Koji Yamamoto, MD, First Department of Internal Medicine, Nagoya University School of Medicine, Tsurumacho, Showa-ku, Nagoya 466, Japan.

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0006-4971/91/7712-0013$3.00/0

DNA ligase (New England Biolabs, Beverly, MA). These subcloned PCR products were then sequenced by the dideoxy chain termination method of Sanger et al[15] using the Sequenase ver.2.0 sequencing kit purchased from United States Biochemical Corp (Cleveland, OH) and [α-35S] deoxyadenosine-triphosphate (Amersham, Arlington, IL). We ruled out the mispolymerization of Taq polymerase by sequencing multiple clones with distinct orientation.

RESULTS

M13 phage clones containing each of nine exons of protein C gene originated from a protein C-deficient subject (patient M.U.) were sequenced by a standard dideoxy termination method. No nucleotide change was found in the exons of the protein C gene, except for one nucleotide deletion in the last exon and one silent mutation (T-G) in exon 6 at the third codon encoding Ser at the 99 amino acid residue; the patient was heterozygous at this site (Fig 1). The nucleotide deletion in the last exon, which leads to a frameshift mutation, was the only difference from a normal coding sequence and was supposed to be responsible for protein C deficiency in this patient (unpublished observation, March, 1990). The detailed analysis of this mutation is now under investigation. In contrast, the single-base substitution in exon 6 does not change a coding information and represents a DNA polymorphism. Because this nucleotide mutation does not create or destroy any known restriction enzyme site, we have introduced A instead of G at nucleotide 3345, which is the third nucleotide 3' to the de novo mutation at nucleotide 3342, by using the designed primers (Fig 2). After PCR, amplified fragments from a more-frequent allele (A1:TCT) should contain the new *Xba* I site (-T/CTAGA-), yielding two fragments (182 bp and 30 bp in length) by the enzyme digestion. A less-frequent allele (A2:TCG) should not have the enzyme site, generating only a 212-bp fragment after *Xba* I digestion. This DNA polymorphism was named PC-493 according to the nucleotide number of the mutation site in protein C cDNA. It occurs at a frequency of 0.18 in the Japanese population (40 chromosomes analyzed), having a 28.9% estimated heterozygosity rate. In Caucasians, a frequency of this sequence polymorphism was 0.25 (20 chromosomes analyzed), and a significant difference did not exist between Japanese and Caucasian populations.

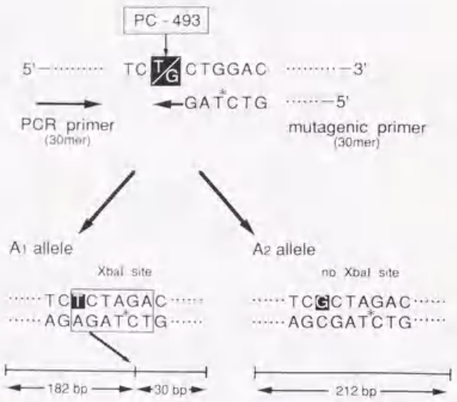

Fig 2. Strategy for direct detection of polymorphic locus PC-493 using mutagenic primers. The polymorphic site (PC-493) was shown as T/G in exon 6 of the protein C gene. We introduced adenine instead of guanine at nucleotide 3345 (shown as T* in the figure). A new *Xba* I site (-T/CTAGA-) is introduced in the amplified fragments from a more-frequent allele (A1:TCT) yielding two fragments (182 bp and 30 bp in length) after the enzyme digestion. A less-frequent allele (A2:TCG) not having the enzyme site generates only a 212-bp fragment by PCR-*Xba* I digestion.

We also investigated the haplotype inter-relationships between PC-493 and the *Msp* I polymorphism reported by Te Lintel Hekkert et al[16] in the Japanese population (54 chromosomes analyzed). The results are shown in Table 1 (B1 allele, not having *Msp* I site; B2 allele, having *Msp* I site). The expected frequencies of each haplotype, if these loci segregated independently, are shown for comparison.

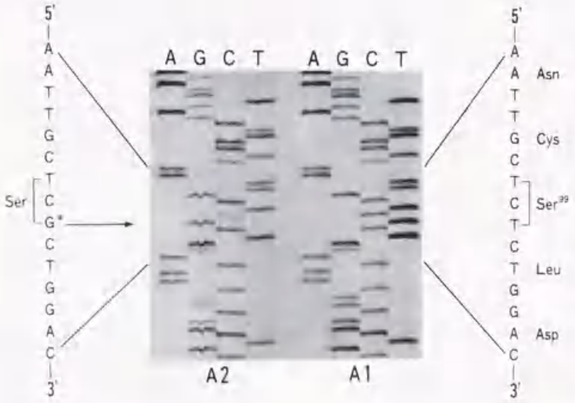

Fig 1. A neutral point mutation in exon 6 of the human protein C gene. Amplified DNAs of the patient were sequenced as described in Materials and Methods. A T to G substitution (G*) at the third codon coding for Ser at the 99-amino acid residue was shown in the A2 allele. A1, more-frequent allele; A2, less-frequent allele.

Table 1. Haplotype Analysis of PC-493 and the *Msp* I Polymorphism

Haplotype			
PC-493	*Msp* I	Observed*	Expected†
A1	B1	7	16
A1	B2	33	24
A2	B1	14	6
A2	B2	0	8

*The genotypes corresponding to the *Msp* I polymorphism and PC-493 were determined in 27 unrelated individuals of the Japanese population.

†The values were calculated from the allele frequencies in the population analyzed.

The frequency of the heterozygotes of PC-493 among the homozygotes of the *Msp* I polymorphism was found to be 0.167 (data not shown). However, the A2 allele of PC-493 was perfectly associated with the B1 allele of the *Msp* I polymorphism to form a preferred haplotype. Hence, it appears that there is some degree of linkage disequilibrium between these two polymorphisms.

A pedigree of the protein C deficiency (patient Y.T.) was analyzed by PC-493 polymorphism (Fig 3). This kindred had three individuals with heterozygous protein C deficiency, including the propositus. A phenotypic protein C-deficient status of the mother (II-2), the elder sister (III-1), and the patient (III-3) was in good concordance with having the A1 allele of PC-493 polymorphism. On the other hand, unaffected members such as the grandmother (I-2), the father (II-1), and the younger sister (III-2) had only an A2 allele of this polymorphism. The grandfather (I-1) who had only an A1 allele of PC-493 polymorphism was found to have normal activity and antigen levels of protein C. These results suggest that the protein C gene of the mother gave rise to the mutation responsible for protein C deficiency in this pedigree.

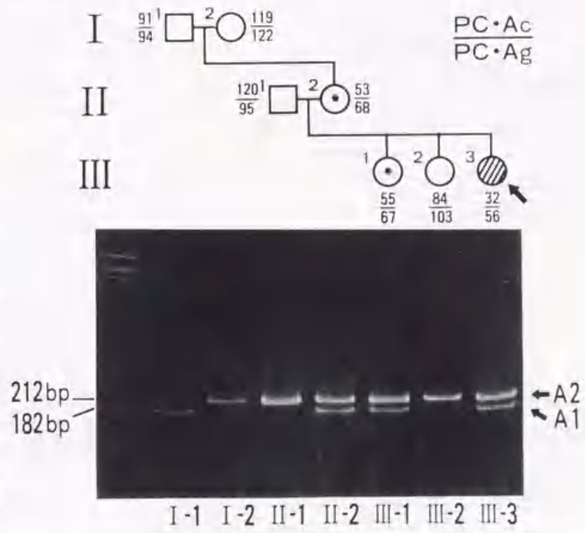

Fig 3. Family diagnosis of the protein C deficiency by PC-493 polymorphism. Ethidium bromide-stained 9% polyacrylamide gel of PCR products after digestion with *Xba* I was shown. Heterozygotes of protein C deficiency are indicated by a dot in circle. The proband is indicated by an arrow. Squares, males; circles, females; hatching, female with clinical thrombotic diseases. Protein C activities are given above the bar, and antigen levels are shown beneath the bar as the percent compared with normal pooled plasma. All protein C-deficient individuals have the A1 allele in the pedigree, except for the grandfather, whose antigen and activity of protein C are within normal range. The A1 allele is linked with the gene responsible for the protein C deficiency in this pedigree. I-1, grandfather; I-2, grandmother; II-1, father; II-2, mother; III-1, elder sister; III-2, younger sister; III-3, patient.

DISCUSSION

Prenatal diagnosis or carrier diagnosis has recently been performed using restriction fragment length polymorphism (RFLP) in various types of inherited blood diseases such as thalassemias,[17,18] hemophilia A,[19] hemophilia B,[20,22] von Willebrand disease,[23] and antithrombin III deficiency.[24] In protein C deficiency, several RFLP sites within or near to the protein C gene have been discovered. Te Lintel Hekkert et al[16] have reported two RFLPs detected by *Msp* I and *Apa* I, respectively, at approximately 7 kb 5' to the first exon of the protein C gene. The frequency of these polymorphisms among the unrelated European Caucasian population was 0.31. *Msp* I RFLP in intron 8 of the protein C gene has been described to have a frequency of 0.01 in the healthy Caucasian population.[25]

To date, no other useful RFLP sites linked to the protein C gene have been discovered. Beckmann et al[8] found a T to C substitution at the third nucleotide coding for Asp at the 214 residue as a genetic variant, suggesting the heterogeneity of the human protein C gene. However, in five individual clones that were completely sequenced in the present study, we have identified only thymidine residue at this locus. This observation implies that variation at Asp-214 may not be frequently found among the Japanese population. The third nucleotide at Ser-99, originally reported as a G in the cDNA of protein C by Beckmann et al,[8] has been intensively investigated and confirmed as the sequence polymorphism (PC-493) in this study. Because the PC-493 locus was not a restriction site, we have applied mutagenic primers and

restriction enzyme digestion to detect this DNA polymorphism.[26] Twenty-nine percent of Japanese individuals and 38% of Caucasians were found to be heterozygous for PC-493 polymorphism.

From the results of a linkage study between the *Msp* I polymorphism and PC-493, PC-493 will be informative in 16.7% of cases that are uninformative for the *Msp* I polymorphism. Therefore, more improvement in the genetic diagnosis of protein C deficiency would be expected by the combined use of these two polymorphisms. We believe that the PC-493 polymorphism is useful for tracing the affected protein C gene among protein C-deficient family members, as we have shown in this study. The polymorphic DNA segment can be amplified and directly tested for the presence of the alternative sequences through a nonradioactive procedure that has the advantage of speed (1 to 2 days). This strategy to detect a single base mutation may be used in wide range of genetic disorders[27] if the information of the mutation is available at the nucleotide sequence level.

ACKNOWLEDGMENT

We appreciate Dr Masanori Awaya, the Sakuragaoka Branch Hospital of the Toyohashi-Shimin Hospital, Toyohashi, Japan, and Dr Kazuo Tomita, Anjo-Kosei Hospital, Anjo, Japan, for providing their patients' materials. We thank P.H. Reitsma, PhD, for providing the pWPC 1 clone and information on the sequence of the primer for detecting the *Msp* I polymorphism. We also thank Takayo Izuhara and Sayoko Sugiura for technical assistance.

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ヒトプロテインC遺伝子における2つの新しい遺伝子多型部位
山本晃士, 高松純樹, 齋藤英彦

Nucleic Acids Research, Vol. 19, No. 24 6973

An *Eco*RI RFLP downstream of the human c-*myc* gene

K.Yamamoto, H.-F.Lin, M.Yamashita, N.Miyoshi+, A.Tojo and M.Shibuya*
Department of Genetics, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108, Japan

Source/Description: 1A4F (D8S197) is a 3.8 kb *Eco*RI genomic fragment mapped more than 100 kb downstream of the c-*myc* gene, and subcloned into pUC119. This probe is derived from cosmid clone # 1A which was obtained by screening a cosmid library of human leukocytes (1) with a DNA fragment of the c-*myc* gene amplicon in GL-16 human glioblastoma cell line. Although we have not precisely determined yet the location of 1A4F, we suggest that its position is between 100 to 300 kb downstream of the c-*myc* gene, since 1A4F is within the amplicon of COLO320 but outside of the amplicon of HL60 (Lin and Shibuya, unpublished results) (2, 3).

Polymorphism: *Eco*RI identifies a two-allele polymorphism, 5.8 kb (A_1) and/or 3.8 kb (A_2).

Frequency: 50 unrelated Japanese individuals were studied.
A_1: 0.18
A_2: 0.82

Not Polymorphic For: *Hin*dIII, *Pst*I, *Kpn*I, *Sac*I, *Bam*HI.

Chromosomal Localization: 8q24.

Mendelian Inheritance: Co-dominant inheritance was observed in three two-generation families (a total of 17 individuals).

Probe Availability: Contact M. Shibuya.

Other Comments: This probe needs to be competed with sheared human placenta DNA because it contains human genomic repeated sequences (4). We are currently isolating a unique sequence from 1A4F clone. (Comments 1 and 2 from 10/30/91 letter).

Acknowledgements: We thank Drs Y. Ueyama, N. Tamaoki, and M. Terada for providing GL-16 human glioblastoma cell line and a human cosmid library. This work was supported by Grant-in- Aid for Special Project Research on Cancer-Bioscience 02262204 from the Ministry of Education, Science and Culture of Japan.

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* To whom correspondence should be addressed
+ Present address Department of Veterinary Pathology, Faculty of Agriculture, Kagoshima University, Kagoshima 890, Japan

Two novel sequence polymorphisms of the human protein C gene

K.Yamamoto, J.Takamatsu and H.Saito
First Department of Internal Medicine, Nagoya University School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466, Japan

Source/Description: A 287 bp fragment (nt 3174–3460 as in ref. 1) amplified as described by Yamamoto *et al.* (2) using the primer D-2' (nt 3431–3460) and the mutagenic primer M-1 (nt 3174–3203, with only one substitution of ^{3201}C→A) for PC-457 detection, and a 134 bp fragment (nt 6078–6211 as in ref. 1) amplified using the primer E-1 (nt 6078–6107) and the mutagenic primer M-2 (nt 6182–6211, with only one substitution of ^{6184}A→T) for PC-664 detection.

Polymorphism: A C→T transversion in the triplet coding for ^{87}Arg (CG**C**:F1→CG**T**:F2), named as PC-457 according to the nucleotide number of the protein C cDNA, and an A→G transition in the triplet coding for ^{156}Lys (AA**A**:G1→AA**G**:G2), named as PC-664 as described above, were found. MaeII (A/GCT) identifies the two sequence polymorphisms when used to digest the 287 bp fragment (F1) (to a 259 bp and a 28 bp fragment (F2)) and the 134 bp fragment (G1) (to a 104 bp and a 30 bp fragment (G2)).

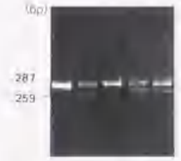
Frequency: Studied in 28 unrelated Japanese individuals
PC-457: F1 = 0.875, F2 = 0.125, heterozygosity rate = 21.9%
PC-664: G1 = 0.643, G2 = 0.357, heterozygosity rate = 45.9%

Chromosomal Localization: The protein C gene has been localized on chromosome 2 (3).

Mendelian Inheritance: Co-dominant segregation shown in 2 families, 12 individuals.

Primer Availability: Available for collaborative studies; write to Koji Yamamoto.

References: 1) Foster,D.C. *et al.* (1985) *Proc. Natl. Acad. Sci. USA* **82**, 4673–4677. 2) Yamamoto,K. *et al.* (1991) *Blood* **77**, 2633–2636. 3) Rocchi,M. *et al.* (1986) *Hum. Genet.* **74**, 30–33.



PC-457

SacI RFLP in the insulin-like growth factor 2 receptor gene (IGF2R) on human chromosome 6q

R.Hauptschein, R.Dalla-Favera* and G.Gaidano
Department of Pathology and Cancer Center, College of Physicians & Surgeons, Columbia University, New York, NY 10032, USA

Source/Description: The probe used for Southern hybridization is a 4.6 kb *Kpn*I fragment derived from the 8.6 kb IGF2R cDNA clone (pSVLhMPR5) (1).

Polymorphism: *Sac*I identifies a two allele RFLP with bands at 18.6 kb (A1) and 14.4 kb (A2). Invariant bands are 5.3 kb, 4.9 kb, 4.0 kb, 3.1 kb and 2.2 kb.

Frequency: Calculated from 31 unrelated Caucasians
A1: 0.65
A2: 0.35
Observed heterozygosity: 0.52.

Not Polymorphic For: *Alu*I, *Bam*HI, *Eco*RI, *Hae*III, *Hin*dIII, *Hin*fI, *Msp*I, *Pst*I, *Pvu*II, *Stu*I, *Taq*I and *Xmn*I (tested on a panel of 9 unrelated individuals).

Chromosomal Localization: IGF2R has been assigned to human chromosome 6q25−27 using somatic cell hybrids and *in situ* chromosomal hybridization (2).

Mendelian Inheritance: Co-dominant segregation demonstrated in 3 informative families.

Probe Availability: Contact Dr William S. Sly, Department of Biochemistry and Molecular Biology, St Louis University School of Medicine.

Acknowledgements: We are grateful to Dr Sly for providing us with the pSVLhMPR5 IGF2R cDNA clone. This work was supported by National Institutes of Health grant CA-44029 (to R.D.F.). G.G. is partially supported by Comitato Gigi Ghirotti.

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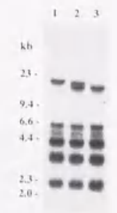

Figure 1. IGF2R RFLP detected by Southern blot analysis of SacI digested human DNA. The two alleles are identified as 18.6 kb (A1) and 14.4 kb (A2) bands in a .8% agarose gel. Lane 1: A1 homozygous; lane 2: heterozygous; lane 3: A2 homozygous. Hybridization and washing conditions were 50% formamide, 3×SSC, 37°C and .2×SSC, 60°C, respectively.

* To whom correspondence should be addressed

A PvuII RFLP at D6S114E locus in the HLA region (RING4 locus)

F.Muntoni, R.Lampis[1], F.Frau[1], L.Argiolas[1], M.R.Murru, G.Costa and M.Congia[1]
Laboratorio di Ematologia e Patologia Molecolare, CNR e [1]Istituto di Clinica e Biologia dell'Età Evolutiva, Via Jenner, 09100 Cagliari, Italy

Source and Description of Clone: The probe P21U is a 2.8 kb cDNA RING4 clone derived from the U937 cDNA library and subcloned in CDM8 (1).

Polymorphism: PvuII identifies a two allele polymorphism with fragments of either 6.8 kb (A1) or 3.9 and 2.9 kb (A2) in length. No other bands are detected.

Frequency: As determined from 76 chromosomes of unrelated subjects of Sardinian origin.
Allele A1 = 0.68
Allele A2 = 0.32

Not Polymorphic For: 9 enzymes including BamHI, BglII, EcoRI, HindIII, MspI, PstI, RsaI, TaqI and XbaI.

Chromosomal Location: The human RING4 gene has been localised to the short arm of chromosome 6, between HLA DOB and DNA genes, about 25 kb centromeric to the former.

Mendelian Inheritance: Mendelian inheritance was demonstrated in three two-generation families tested.

Other Comments: No linkage disequilibrium between the two RING4 alleles and any of the 21 DPB1 or the 9 DQA1 HLA genes was found. Moreover no linkage disequilibrium between any of the two peculiar HLA extended haplotypes present in Sardinia at a very high frequency (2, 3), namely A30, B18, Cw5, DR3, DRw52, DQw2 and A2, Cw7, B17, 3F31, DR2, DQw1, and the two RING4 alleles was observed.

Probe Availability: Contact Dr John Trowsdale, Imperial Cancer Research Fund, Lincoln's Inn Fields, London.

Acknowledgements: We thank Dr John Trowsdale for the kind donation of the probe.

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