Detection of Single Nucleotide Polymorphisms in Coagulation Factor XI Deficient Patients by Multitemperature Single-Strand Conformation Polymorphism Analysis

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Factor XI (FXI) deficiency is a rare inherited disorder which can cause bleeding complications especially in case of hemostatic challenge and/or in tissues with high fibrinolytic activity. A number of causative mutations have been described in FXI deficient individuals which have been detected by various screening methods. In this study, we present the application of the multitemperature single-strand conformation polymorphism analysis (MSSCP) on the FXI gene, a recently developed methodology for the detection of single nucleotide exchanges. We analyzed a total of 217 polymerase chain reaction (PCR) fragments from the promoter region as well as from exons 1–7 and 11–15 and compared the results to automatic fluorescent sequencing. A total of 29 PCR fragments showed single nucleotide exchanges in

conventional fluorescent sequencing, representing 10 different mutations (nine missense mutations, one small deletion) and four frequent polymorphisms. With MSSCP electrophoresis at a standard temperature profile (gel temperature 35–20–10°C) we were able to detect 13 of 14 (93%) different nucleotide exchanges in 25 of 29 PCR fragments (86%). Hence, the detection rate for genetic variations in the FXI gene was 86%. To evaluate the reproducibility, MSSCP was performed twice for 174 PCR fragments and the consistency between two electrophoretic runs was 99%. We conclude that the MSSCP is a sensitive, fast, and cost effective screening method for the detection of FXI gene mutations. *J. Clin. Lab. Anal.* 19:233–240, 2005. © 2005 Wiley-Liss, Inc.

Key words: factor XI deficiency; mutation; SSCP; MSSCP

INTRODUCTION

FXI is the zymogen of a serine protease that, upon activation to FXIa by small amounts of thrombin on the surface of platelets, promotes coagulation by the additional generation of thrombin. This activates the thrombin-activatable fibrinolysis inhibitor which allows clot stabilization by decreasing sensitivity to fibrinolysis (1).

FXI is synthesized in the liver and circulates in the blood as a homodimer of two identical polypeptides linked by a disulfide bond in a noncovalent complex with high molecular weight kininogen (2).

On activation, FXI is cleaved at the Arg369-Ile370 polypeptide bond, yielding a heavy and a light chain. The light chain contains the catalytic domain of the enzyme and is homologous to the trypsin family of

serine proteases. The heavy chain is composed of four tandem repeats, the so-called apple domains, held together by 3 characteristic disulfide bonds (3). The binding sites for high-molecular weight kininogen, thrombin and prothrombin, a site necessary for the Ca²⁺ dependent activation of coagulation factor IX as

Abbreviations: FXI, coagulation factor XI; SSCP, single-strand conformation polymorphism; MSSCP, multitemperature single-strand conformation polymorphism analysis; PCR, polymerase chain reaction.

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Received 21 February 2005; Accepted 17 June 2005

DOI 10.1002/jcla.20084

Published online in Wiley InterScience (www.interscience.wiley.com).

well as a binding site for platelets in the presence of high-molecular weight kininogen are localized to the heavy chain. This chain is also involved in the dimerization and secretion of FXI (4–9).

The human FXI gene is located on the long arm of chromosome 4 (4q35) (10) spreading over approximately 50 kb (11). While exons 3–15 encode for the mature protein, exon 1 encodes for the 5′ untranslated region and exon 2 for a signal peptide which is not integrated in the mature protein (12). Apart from the type II (Glu117Stop) and type III (Phe283Leu) mutations, which cause a high percentage of inherited FXI deficiency in the Jewish population (12,13), increasing numbers of causative mutations have been identified in different ethnic groups.

Because of the heterogeneity of mutations and the size of the FXI gene, an efficient mutation screening technique is required for the molecular diagnosis of FXI deficiency. One of the most widely used methods for the detection of single nucleotide polymorphisms in polymerase chain reaction (PCR) fragments is the single-strand conformation polymorphism (SSCP), a simple, cheap, and time-saving method (14). Single-stranded DNA fragments (ssDNA) with single nucleotide exchanges can be distinguished by means of SSCP on the basis of their different electrophoretic mobility in polyacrylamide gels under native conditions as they show conformational isoforms (15,16). However, the detection rate of the SSCP is approximately 80–85% (17), and some three-dimensional ssDNA isoforms can only be separated under specific conditions. The most important variables for separation are ionic strength, pH, and temperature (18,19). In particular, electrophoresis often has to be repeated at different temperatures to improve the detection rate. This leads to an increase in costs and analysis time.

In this study, we present the application of a recently developed method, the multitemperature single-strand conformation polymorphism (MSSCP) (20), for mutation screening in the FXI gene. In the MSSCP analysis, different temperatures are applied during a single electrophoretic run at native conditions to improve the detection rate of point mutation discovery.

Results of MSSCP electrophoresis were compared to automatic fluorescent sequencing data to establish the MSSCP detection rate for sequence variations in the FXI gene. Furthermore, the interexperiment reproducibility of MSSCP was evaluated by performing repetitive runs.

MATERIALS AND METHODS

Patients

The 20 studied patients attended hospital for reasons other than bleeding disorders. They underwent routine

laboratory tests and were selected because they showed prolonged activated partial thromboplastin time (APTT). Patients were then evaluated for coagulation disorders and those who showed no other coagulation abnormality than FXI deficiency were included in the study. APTT and assays for factor activities were performed in duplicate on an STA coagulation analyzer using specific one stage clotting assays (Diagnostica Stago, Asnieres-sur-Seine, France).

DNA Isolation and PCR

Genomic DNA was isolated from peripheral blood leukocytes using the protocol of the ViennaLab Gene Mutation Assays (Vienna Lab Labordiagnostika GmbH, Vienna, Austria) according to the manufacturer's recommendations. For PCR amplification of DNA, oligonucleotide primers were used as described (21,22), amplifying exons 1–7 and 11–15 and the exon–intron boundaries. For the amplification of the promoter region, new oligonucleotide primers were designed (Table 1).

The PCR reaction was performed on an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) using 3 μ L of matrix DNA, 0.2 mM of dNTPs (Finnzyme, Espo, Finland), 20 pmol of forward and reverse oligonucleotide primers in 1 $\times$ PCR Taq Polymerase buffer (Roche, Mannheim, Germany) and 1 Unit Taq Polymerase (Roche, Mannheim, Germany). Details of PCR amplifications are presented in Table 2. Generally, PCR was started with a 3-min denaturation step at 95°C. After 35 cycles of amplification, 5 min of elongation at 72°C finalized the PCR reaction.

Aliquots of the PCR products were visualized by electrophoresis on 2.5% agarose gels to control the specificity of the PCR reaction.

MSSCP Analysis

A total of 4 μ L of PCR product were diluted in 6 μ L of denaturing buffer A for MSSCP (Kucharczyk T.E., Warsaw, Poland) and heated at 55°C for 10 min. Afterward, the samples were chilled on ice and 2 μ L of denaturing buffer B for MSSCP were added.

The samples were then analyzed on 9% polyacrylamide gel for MSSCP analysis (Kucharczyk T.E., Warsaw, Poland) prepared with 3.3% bisacrylamide and 4.5% glycerole in 0.75 $\times$ Tris-Borat-EDTA (TBE) buffer.

The MSSCP conditions were kept identical for all analyzed PCR fragments: after 10 min of preelectrophoresis with constant 40 W power the samples were loaded on the gel. Initial electrophoresis with 100 V for 10 min was performed to allow samples to enter the gel. Next, 40 W constant power electrophoresis was applied

TABLE 1. Oligonucleotide primers used for amplification of FXI gene

Region of FXI gene	Forward primers	Reverse primers
Promotor	5'- CTC TTA GGA GCT GTC TGT ACT T - 3'	5'- GTG TAG GAA ATG GTC ACT TCA C - 3'
Exon 1	5'- AGC AAT TCT CTC AAG G - 3'	5'- GCG GAA CAT CTC TAC AAA GC - 3'
Exon 2	5'- TTA ACT ACT CCC CTC TCT CCC TAT T - 3'	5'- CCT CAA TTC CTA CCC TGC TTT GAA - 3'
Exon 3	5'- CCC AGT AAA ATC CAA CAT AAC GC - 3'	5'- GTC TCC TCG ATG TAG AAA CAT AAG - 3'
Exon 4	5'- TTC TTT TTG GCT TTC TGT GTG CTG - 3'	5'- GAA CAT ATC TTA CCG CTT ATT TGG TG - 3'
Exon 5	5'- TCT GGA AGG TAC TCA TGT CTT CTG - 3'	5'- TTC ATC GAC CAC TCG AAT GTC CTG - 3'
Exon 6	5'- ACA TTT GTC TAC TGA AGC ACA CCC - 3'	5'- GGT CCG TTT CAT CGT GAG CAT AAG - 3'
Exon 7	5'- GAT AGC TGG TGA ATT GAG TCC CTG - 3'	5'- CCT TAC CTT TGA GAT TCT TTG GGC - 3'
Exon 11	5'- TGC TTC TGT TGC AGA GTG TAC C - 3'	5'- GAT GAA CTA ATA AAA ACA GCC GTG G - 3'
Exon 12	5'- TCT TTC CCT CTG TTG TTT GCT C - 3'	5'- TCC GTA CCT GTG TAA TTC ACT G - 3'
Exon 13	5'- AGA GTC TCT TCT GGA AAA GAG G - 3'	5'- TTC TAG GAT GGA GCA CAT ATA ACA AC - 3'
Exon 14	5'- CAA AAT ACT CTC CAG AAA GCC AAG - 3'	5'- CAA AAA AAC CCC AAC GCA TTA AGC - 3'
Exon 15	5'- CTT TTC TTG TCT CCC CTC GTT - 3'	5'- GAC TGA ATG CTC AAA CAA ACT ACA AC - 3'

TABLE 2. Polymerase chain reaction conditions*

Exon	Denaturation	Time	Annealing	Time	Elongation	Time
1	94°	20	53°	30	72°	40
2	94°	20	59°	30	72°	40
3, 4, Promotor	94°	20	61°	30	72°	40
5	94°	20	55°	30	72°	40
6, 7	94°	20	63°	30	72°	40
11, 12, 13, 15	94°	20	58°	30	72°	40
14	94°	20	62°	30	72°	40

*Temperature is given in ° Celsius, time in seconds.

for 45 min at a gel temperature of 35°C, followed by electrophoresis with 40 W power for 40 min at 20°C, and then for 35 min at 10°C. Electrophoresis was performed in a DNA Pointer System (Kucharczyk T.E., Warsaw, Poland) with 0.1°C gel temperature control during electrophoresis.

After electrophoresis, gels were stained with the Silver Stain Kit (Kucharczyk T.E., Warsaw, Poland) and dried. The dried gels were scanned at 300 dpi and analyzed using the Gel Scan Software (Kucharczyk T.E., Warsaw, Poland).

Sequencing

All amplified regions were sequenced by automatic fluorescent sequencing, using the 3100 Genetic Analyzer (Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit, Version 2.0; Applied Biosystems, Foster City, CA) or the Long READIR 4200 Analyzer (SequiTherm EXCEL II DNA Sequencing Kit-LC for 25–41-cm gels; LI-COR, Lincoln, NE).

Consistency Evaluation of MSSCP Results

For 174 PCR products the MSSCP experiments were performed twice. The consistency of the results was

evaluated as $[(T-D)/T] \times 100\%$, where T is the total number of twice-analyzed PCR products and D is the number of samples with different MSSCP patterns.

RESULTS

The majority of the 217 analyzed fragments did not show any single nucleotide alteration in the MSSCP analysis or in the automated fluorescent sequencing. In 29 samples a total of 14 different single nucleotide exchanges was detected by automatic fluorescent sequencing (Table 3). A total of nine caused an amino acid substitution in an exon, another one was a small deletional mutation resulting in a frameshift and premature stop codon. The other four detected exchanges comprised a frequent T>G exchange in the promoter region, a silent nucleotide exchange in exon 3 (G>T, Val3silent), and frequent polymorphisms in exons 5 and 15 (C>T, Asp125silent; and T>C, Gly379silent).

During the MSSCP electrophoresis at the particular temperature conditions chosen, 25 of the 29 fragments showed different electrophoretic mobility patterns compared to the wild-type PCR fragments. Four results were false negative (Table 3). In sample 73, the T>G

TABLE 3. Results of genetic diversity detection in FXI gene and comparison of MSSCP results with the sequencing results*

Gene region	Sample	MSSCP 1	MSSCP 2	Sequencing results
Promotor	64	+	nd	Polymorphism ^a
	71	+	nd	Polymorphism ^a
	72	+	nd	Polymorphism ^a
	73	–	nd	Polymorphism ^a
	75	+	nd	Polymorphism ^a
	76	+	nd	Polymorphism ^a
	84	+	nd	Polymorphism ^a
	94	+	nd	Polymorphism ^a
Exon 3	50	+	+	Mutation
	72	–	–	Polymorphism ^b
	75	–	–	Polymorphism ^b
	80	–	–	Polymorphism ^b
Exon 4	72	+	+	Mutation
Exon 5	40	+	nd	Mutation
	44	+	nd	Mutation
	75	+	+	Polymorphism ^c
	81	+	nd	Mutation
Exon 6	44	+	nd	Mutation
	52	+	nd	Mutation
Exon 7	66	+	nd	Mutation
	91	+	nd	Mutation
Exon 11	50	+	+	Mutation
	64	+	+	Polymorphism ^d
	71	+	+	Polymorphism ^d
	73	+	+	Polymorphism ^d
	75	+	+	Polymorphism ^d
	82	+	+	Polymorphism ^d
	88	+	+	Polymorphism ^d
Exon 14	91	+	+	Polymorphism ^d
	72	+	–	Wild type

^a–267 C>T.^bVal3silent.^cAsp125silent.^dGly379silent.

nd, MSSCP analysis has not been repeated for this sample.

*Only data of analyzed fragments that revealed any kind of single nucleotide exchange are included. Exons where no exchanges in any of the analyzed samples were detected are not listed. The terminus polymorphism is used in the sense of frequent single nucleotide exchanges without consequent changes in the amino acid sequence (non disease causing change). The terminus mutation stands for single nucleotide exchanges which result in an amino acid substitution (disease causing change).

exchange in the promoter region could not be detected. In samples 72, 75, and 80, the MSSCP of exon 3 showed a thick band in the first analysis indicating nonseparation of two bands (Fig. 1A and B). However, when the analysis was repeated with a lower number of PCR products (0.5 µL) the mobility patterns were then identical to the wild-type samples. Consequently, we were unable to detect two frequent polymorphisms found by fluorescent sequencing in these samples.

Apart from these four false-negative results, MSSCP was able to detect clearly and reproducibly all nine single nucleotide exchanges causing amino acid sub-

stitutions, the small deletional mutation as well as 3 of 4 polymorphisms. We were able to detect 13 of 14 (93%) different nucleotide exchanges in 25 of 29 PCR fragments (86%). Hence, the detection rate for genetic variations in the FXI gene was 86%. MSSCP patterns of samples from exon 5, exon 6, and exon 7 are shown in Fig. 2A and B–D.

Exon 14 of sample 72 produced a false-positive result in one of the MSSCP experiments. Hence, one sample out of 174 analyzed in duplicate produced different motility patterns in two electrophoretic runs. We therefore estimated the MSSCP reproducibility to be 99% = [(174–3)/174] × 100%. The rate of false-positive results was estimated to be below 1% (one sample of total 363 PCR fragments analyzed).

The overall analysis time for 36 PCR fragments was less than 3 hr, the estimated cost of analysis was 0.3 Euro per PCR fragment.

DISCUSSION

FXI deficiency is a rare inherited disorder which can cause bleeding complications, for instance in case of hemostatic challenge and especially in tissues with high fibrinolytic activity (12). Apart from the type II and type III mutations, which cause a high percentage of inherited FXI deficiency in the Jewish population (12,13), increasing numbers of causative mutations have been identified in different ethnic groups by various genetic screening methods. For the purpose of mutational analysis, the use of automated fluorescent sequencing (11,23–26), SSCP (27), dideoxyfingerprinting (22) and denaturing high-performance liquid chromatography (17) have been described. We report the application of the recently developed multitemperature single-strand conformation polymorphism analysis (MSSCP) using the DNA Pointer System.

An influence of temperature on SSCP analysis has been reported by several studies (28–30). Presently, there is no theoretical model which allows to optimize conditions for SSCP analysis. The optimal gel temperature is selected on basis of empirical data. Although SSCP analysis is generally regarded fast and cost-effective, the need to optimize separation conditions increases costs and analysis time. We have previously shown that, changing the gel temperature during the native SSCP electrophoresis as performed in the MSSCP, results in a higher detection rate of point mutations, significantly shorter time of analysis and lower overall costs of mutation detection in comparison to conventional single temperature SSCP analysis (20). We assume that if two different ssDNA isoforms are present, changes in the gel temperature during electrophoresis do increase the probability that they will fold

Fig. 1. **A:** Results of MSCP analysis of PCR products from Exon 3. K: wild-type samples; 50–94 patients' samples. Asterisks on the left side of the line indicate bands with altered mobility in sample 50 and background in samples 72 and 80. For samples 72, 75, and 80, the MSCP of exon 3 showed a thick band which could represent nonseparation of two bands in the first analysis. We therefore repeated the analysis with a lower amount of PCR products. The mobility patterns were then identical to the wild-type samples. **B:** Densitogram plot for A. Arrows indicate the additional peak in sample 50 compared to the control sample and the background in sample 72.

into different mutation-dependent structures and can be distinguished based on their electrophoretic mobility rate. Besides, the DNA Pointer System for MSCP analysis improves temperature control and gel preparation because up to four gels can be prepared simultaneously.

In a single MSCP electrophoresis with the gel temperatures 35°C, 20°C, and 10°C we were able to detected 13 of 14 single nucleotide exchanges found by conventional fluorescent sequencing. Nine were point mutations, changing the amino acid sequence of the FXI gene, and one was a small deletion. All these mutations are likely to affect protein function. The other four exchanges were frequent polymorphisms.

With the electrophoresis conditions used for our experiments we had difficulties in detecting a frequent single nucleotide exchange in the promoter region of sample 73 and another one in exon 3 of samples 72, 75, and 80, which did not produce clearly altered MSCP patterns. For samples 72, 75, and 80, the MSCP of exon 3 showed a strong background in the first analysis

(Fig. 1A and B). We therefore repeated the analysis with a lower amount of PCR product. The mobility patterns were then identical to the wild-type samples and we were unable to detect the Val3silent exchange found by fluorescent sequencing in samples 72, 75, and 80. MSCP did not reliably detect polymorphisms that were exchanges between G and T, while exchanges between C and T in exons 5 and 11 could easily be detected. It has been shown several times that a single-strand DNA mobility in native polyacrylamide gel depends on several physical conditions of electrophoresis, in particular the temperature applied (18–20). We assume that especially for the detection of the silent G>T exchange resulting in the exon 3 Val3silent polymorphism further MSCP optimization by application of different gel temperature profiles could be more successful.

In contrast, all gene alterations leading to amino acid substitutions as well as the small deletional mutation were detected clearly and reproducibly (Fig. 2A and B–D). As these sequence variations lead to structural

Fig. 2. **A:** Result of MSCP analysis of PCR products from Exon 5, Exon 6, and Exon 7. Exon 6: K7, K8, K9, and K10, wild-type samples; 44 and 52, patients' samples. Exon 5: K1, K2, K4, K7, K8, K9, and K10, wild-type samples; 40, 44, and 81, patients' samples. Exon 7: K6, K7, K8, K9, and K10, wild-type samples; 66 and 91, patients' samples. Asterisks on the left sides of the lines indicate altered mobility of samples 44 and 52 in Exon 6, samples 40 and 44 in Exon 5, and samples 66 and 91 Exon 7. **B:** Densitogram plot to Exon 6 in A. Arrows indicate an additional peak in sample 44 and a changed mobility pattern in sample 52. **C:** Densitogram plot to Exon 5 in A. Arrows indicate an additional peak in sample 44 and a double peak in sample 40. **D:** Densitogram plot to Exon 7 in A. Arrows indicate an additional peak in sample 91 and an altered mobility pattern in sample 66.

alterations of the FXI gene, they are likely to be disease-causing changes which have clinical relevance. However, the overall detection rate of MSSCP for all genetic variations found by fluorescent sequencing was 86%. This is at least as sensitive as the conventional "one temperature" SSCP, but with an improved temperature control as well as a more convenient handling in the gel preparation. Furthermore, the total time of analysis up to 36 PCR products is only 3 hours, including sample denaturation and silver staining after electrophoresis. The estimated cost of analysis is as low as 0.3 Euro per PCR fragment.

To estimate the consistency of the MSSCP analysis for different electrophoretic runs in the DNA Pointer System, most analyses were performed twice. We observed an inconsistent result only in sample 72 from exon 14 in a total of 174 samples analyzed twice. This equals to a 99% reproducibility and indicates that the MSSCP in the DNA Pointer System is a method with high precision and reliability for the mutation screening of the FXI gene.

Technical parameters of genotyping methods should always be considered when choosing a screening technology. A screening technique with high sensitivity, reproducibility and short time of analysis is the denaturing high-performance liquid chromatography (dHPLC) with a reported single nucleotide detection rate of 100% (17). However, equipment for dHPLC is expensive in comparison to SSCP or MSSCP analysis and the reproducibility and time effectiveness of MSSCP electrophoresis are comparable to dHPLC.

We conclude that the MSSCP analysis is at least as sensitive as the common "one temperature" SSCP electrophoresis and more cost effective than dHPLC. Furthermore, MSSCP analysis is a time-effective technique for the detection of sequence variations in the FXI gene with high precision and reliability. Screening for sequence variations with MSSCP analysis can significantly reduce the cost of mutation detection by selecting only those samples for conventional sequencing which show an abnormal mobility pattern in the MSSCP electrophoresis.

ACKNOWLEDGMENT

The DNA Pointer System as well as the consumables for the DNA pointer were kindly provided by Kucharczyk Techniki Elektroforetyczne, Warsaw, Poland.

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