Evaluation of Running Conditions for SSCP Analysis: Application of SSCP for Detection of Point Mutations in the LDL Receptor Gene

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We have performed analyses of single-strand conformation polymorphisms (SSCP) of the promoter region and the translated parts of the 18 exons of the low-density lipoprotein receptor (LDLR) gene. DNA from 20 unrelated familial hypercholesterolemia (FH) patients was studied. Four different running conditions were used for the nondenaturing gel electrophoresis to systematically evaluate how differences in the running conditions affect the sensitivity of the assay. These conditions were 15 W, 40 W, and 50 W in the absence of glycerol, and 50 W in the presence of 10% glycerol. SSCP analyses of the 18 PCR fragments for the 20 subjects revealed a total of 46 genotypes at 15 W, 45 at 50 W, 42 at 40 W, and 41 at 50 W with 10% glycerol. A total of 53 different genotypes were observed when the results of the four conditions were considered together. Assuming that the four conditions together detected 100% of the different genotypes, the sensitivity of the four individual conditions ranged between 87% (15 W) and 77% (50 W with 10% glycerol). There were marked differences among the different running conditions to detect abnormal SSCP patterns of individual exons. Therefore, different conditions should be used for the different exons of the LDLR gene.

Analysis of single-strand conformation polymorphisms (SSCP)⁽¹⁻³⁾ has become an important tool for detecting point mutations underlying genetic diseases. This technique is based on a single point mutation altering the conformation of single-stranded DNA (ssDNA). Altered conformation affects the migration of ssDNA that can be detected as abnormal bands on a nondenaturing gel. The mutations underlying the abnormal bands are then characterized by DNA sequencing.

The sensitivity of SSCP in detecting point mutations is dependent on several factors. These include the length of the fragment, the extent of cross-linking concentration of acrylamide, the presence of glycerol, the temperature during electrophoresis, and buffer concentration.^(3,4) Thus, it is of great importance to finetune the SSCP assay.

The most important factor in this respect is the temperature during electrophoresis. This is because the formation of intrastrand bonds, and thereby the conformation of ssDNA, is temperature dependent. The temperature during electrophoresis can be altered by altering the ambient temperature or by altering the wattage. In addition to temperature, the presence of glycerol in the gel may affect the conformation of ssDNA significantly.^(1,3)

Familial hypercholesterolemia (FH) is an autosomal dominant disease characterized by hypercholesterolemia and premature coronary heart disease.⁽⁵⁾ FH is caused by a mutation in the gene en-

coding the low-density lipoprotein receptor (LDLR). The LDLR gene consists of 18 exons, the translated parts of which range from 78 to 381 bp.^(6,7) Thus, the gene lends itself to amplification of individual exons by PCR and analyses of SSCP.

In this study we have established the conditions for amplification of the promoter region and the translated parts of the 18 exons of the LDLR gene. Subsequently, SSCP analyses were performed of these PCR fragments from 20 unrelated FH patients. Four different conditions were used for the nondenaturing gel electrophoresis to systematically evaluate how differences in the running conditions affect the sensitivity of the assay.

MATERIALS AND METHODS

Subjects

Twenty unrelated, Norwegian FH subjects were included in the study; 18 were heterozygotes, and 2 were homozygotes. FH was diagnosed on a clinical basis according to the criteria of Goldstein and Brown.⁽⁵⁾ Haplotypes at the LDLR locus were constructed using the seven haplotype markers described by Rødningen et al.⁽⁸⁾ Hypercholesterolemia segregated with haplotypes at the LDLR locus in the families of all 20 subjects (data not shown). None of the subjects possessed the apoB 3500 mutation,⁽⁹⁾ as determined by the assay of Hansen et al.⁽¹⁰⁾ (data not shown).

Amplification of Individual Exons of the LDLR Gene

The primers used to amplify individual exons from genomic DNA were those described by Leitersdorf et al.⁽¹¹⁾ except from amplification of exons 1 and 18. A 343-bp fragment comprising exon 1 and the 5'-flanking region to position -251, was amplified using the primers SP-PROM (5'-TGGAGTGGGAATCAGAGCTTCACGG-3') and SP91.⁽⁹⁾ A 173-bp fragment of exon 18 was amplified using the primers SP150⁽⁹⁾ and SP2684 (5'-ATAAACAAGCTCTGGCAGGCAAT-3'). The mixtures prepared for the PCRs contained 400 ng of DNA, 20 pmoles of each of the two exon-specific primers, 200 μ M dNTPs, 3.3 nM [³²P]dCTP (3000 Ci/mole), and the buffer [10 mM Tris-HCl, 1.5 mM MgCl₂, 50 mM KCl, 0.1 mg/ml of gelatin (pH 8.3)] supplied with the *Taq* DNA polymerase purchased from Boehringer Mannheim (Mannheim, Germany). The reaction volume was 100 μ l. A DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, CT) was used for the PCRs. The conditions for amplification of individual exons were established and are shown in Table 1.

Analysis of SSCP

Eight microliters of the loading buffer (95% formamide, 20 mM EDTA, 0.05% Bromphenol blue, 0.05% xylene cyanol) was added to 2 μ l of radiolabeled PCR products and heated for 2 min at 94°C. A

2- μ l aliquot of this mixture was loaded into each well of a 6% nondenaturing sequencing gel (acrylamide/bisacrylamide, 100:1.5). The sequencing apparatus was a Sequi-gen Sequencing Cell (Bio-Rad, Richmond, CA). The PCR products from each individual were run under four different conditions in a cold room. These conditions were 15, 40, or 50 W in the absence of glycerol and 50 W in the presence of 10% glycerol. The electrophoreses were performed for approximately 1.5 hr at 40 and at 50 W in the absence of glycerol. For the two other conditions the electrophoreses were performed for ~3 hr. The gels were transferred to Whatman 3 MM papers, dried, and autoradiographed for 5–7 days at -80°C.

Evaluation of the SSCP Patterns

All SSCP analyses were performed by one of us (K.S.). Each autoradiogram contained the analyses of one exon for the 20 subjects using one of the four different running conditions. The typing of the band patterns was performed as a two-step procedure. First, two of us (O.K.R. and T.P.L.) typed each running condition for all exons blindly with respect to the results obtained with the three other conditions. Thus, this typing would mimic the situation as if only one running condition were employed. Second, all four running conditions were considered together by three of us (K.S.,

O.K.R., and T.P.L.) when typing each exon for abnormal band patterns. This would represent the situation of employing four different running conditions to screen for mutations.

RESULTS

Pilot Study

Before the systematic SSCP analyses of the 18 exons were started, a pilot study was performed to determine the appropriateness of the four different running conditions that were subsequently used for the study. In the pilot study, three exons (8, 13, and 14) of the LDLR gene containing known mutations were amplified from the DNA of 20 FH subjects. Exons 8 and 13 contain polymorphic *Stu*I⁽¹²⁾ and *Ava*II⁽¹³⁾ sites, respectively. These RFLPs had been analyzed by restriction cutting of amplified PCR fragments. The two patterns observed by SSCP analyses of exon 8 were in accordance with homozygosity or heterozygosity for the presence of the *Stu*I cutting site. These patterns could easily be identified by any of the four conditions used for the gel electrophoresis. The three patterns observed by SSCP analyses of exon 13 were in accordance with the three genotypes of the *Ava*II RFLP. Although the three patterns of exon 13 could be identified by all four running conditions, 15 W was very much superior to the three others (data not shown). One subject who was heterozygous for a mis-

TABLE 1 Fragment Lengths of the Promoter Region and the Translated Parts of the 18 Exons the LDLR Gene and Conditions for the PCRs

	Exon																	
	promoter +1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Fragment length ^a	343	173	173	431	173	173	170	176	222	278	169	190	192	203	221	128	208	173
Primers																		
5'	SP-PROM ^b	SP57	SP59	SP61	SP62	SP64	SP66	SP68	SP70	SP72	SP74	SP76	SP78	SP80	SP82	SP84	SP86	SP150
3'	SP91	SP58	SP60	SP51	SP63	SP65	SP67	SP69	SP71	SP73	SP75	SP77	SP79	SP81	SP83	SP85	SP87	2684 ^b
<i>Taq</i> DNA polymerase (units)	1.5	2	1.5	1	1	0.75	1	1	1	1	1	1	1	1.25	1	1	1	1.5
Thermal cycling																		
denaturation (°C)	94	94	94	95	94	94	94	94	94	94	94	94	94	94	94	94	94	94
annealing (°C)	48	53	50	62	58	45	58	49	55	55	50	50	50	55	50	50	50	50
extension (°C)	72	72	72	72	72	72	72	72	72	72	72	72	72	72	72	72	72	72

All thermal cyclings consisted of 25 cycles. They were preceded by initial denaturation for 7 min at 94°C and completed by terminal extension for 10 min at 72°C.

^a Fragment lengths (in bp) include the two 25-bp exon-flanking oligonucleotides.

^b Primer sequences are given in Materials and Methods. All other primer sequences are from Leitersdorf et al.⁽⁹⁾

sense mutation in codon 664 of exon 14 as determined by restriction cutting with *Pst*I,⁽¹⁴⁾ possessed a SSCP pattern different from those who were homozygous for the wild-type allele. The abnormal pattern in this subject could be identified using any of the four conditions for the gel electrophoresis.

This pilot study told us that we were able to detect all three known mutations and the resulting seven genotypes present in this group of patients. Furthermore, the finding that some genotypes were much easier to detect by one condition than others indicated that running the gel under different conditions would increase the overall sensitivity of the assay.

Applying Single Running Conditions for Gel Electrophoresis

The promoter region and each of the 18 exons of the LDLR gene amplified from 20 FH subjects were examined by SSCP analysis using the four different conditions for the gel electrophoresis. Each condition was analyzed blindly with respect to the results obtained with the three other conditions. The number of different patterns for the four different conditions are shown in Table 2.

There were relatively large differences in the number of different SSCP patterns observed for each exon using the different running conditions. These ranged from 1 (exons 1, 6, 9, 17) to 5 (exons 10–12). No additional information was obtained for seven of the exons (exons 1, 6–9, 17, 18) using four different conditions for the gel electrophoresis as compared with only one. However, for the

other 11 exons there was a marked difference in the sensitivity of the different conditions to detect abnormal patterns. This was most prominent for exon 2, where gel electrophoresis at 40 W revealed the same pattern in all 20 subjects; gel electrophoresis at 50 W in the presence of 10% glycerol, however, revealed four different patterns (Fig. 1). Thus, three additional patterns were observed at 50 W in the presence of 10% glycerol as compared with the pattern observed at 40 W. For exons 4, 10, 11, 14, and 15, the condition with the highest number of patterns had two more patterns than the condition with the lowest number.

The different sensitivities of the running conditions to detect mutations are illustrated in Figure 1. Whereas, 15 W detects three of four different genotypes in exon 2, it detects only one of three genotypes in exon 4. Furthermore, 50 W in the presence of 10% glycerol detects all four genotypes of exon 2 but only two of the three in exon 4.

Applying Four Running Conditions for Gel Electrophoresis

Except for exons 11 and 14, the number of patterns observed with the running condition that had the highest number of patterns was identical to the number observed when the four conditions were considered together. For exons 11 and 14, the condition with the highest number of patterns failed to identify an abnormal band pattern that could be detected with one of the other conditions. Assuming that the number of different patterns represents the number of differ-

FIGURE 1 SSCP analyses of exons 2 and 4 of the LDLR gene were performed using four different running conditions for the nondenaturing gel electrophoresis. These conditions were 15, 40, and 50 W in the absence of glycerol and 50 W in the presence of 10% glycerol. The SSCP patterns of subjects A and B were identical for three of the conditions but were different at 50 W in the presence of glycerol. The SSCP patterns of subjects B–D were identical at 40 W but could be separated using the three other conditions. The SSCP patterns of subjects E and F were identical at 15 W. Subject E was clearly different from F and G with the three other conditions. Subjects F and G were only different at 50 W in the absence of glycerol.

ent genotypes, analyses of SSCP of all 18 PCR fragments for the 20 subjects revealed a total of 46 genotypes at 15 W, 45 at 50 W, 42 at 40 W, 41 at 50 W with 10%

TABLE 2 Observed SSCP Patterns

Condition	Exon																	
	promoter +1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
15 W	1	3	4	1	1	1	2	2	1	5	5	5	3	4	3	1	1	3
40 W	1	1	4	2	2	1	2	2	1	4	5	5	3	3	1	1	1	3
50 W	1	3	4	3	1	1	2	2	1	5	5	4	3	3	1	2	1	3
50 W + 10% glycerol	1	4	3	2	1	1	2	2	1	3	3	5	3	2	3	1	1	3
Total	1	4	4	3	2	1	2	2	1	5	6	5	3	5	3	2	1	3

Number of different SSCP patterns observed for analyses of the promoter region and the 18 exons of the LDLR gene from 20 FH subjects using four different conditions for the gel electrophoresis. These conditions were 15, 40, and 50 W in the absence of glycerol and 50 W in the presence of 10% glycerol. The number of different SSCP patterns shown for each condition is the number observed when the investigators were blinded with respect to the band pattern obtained with the three other running conditions. The total number of patterns is the number observed when all four conditions were considered together.

glycerol, and 53 genotypes when all four conditions were considered together (Table 2).

Assuming that the sensitivity of the four conditions together to detect mutations is 100% (i.e., 53 different genotypes), the sensitivity of each of the four conditions is 87% (15 W), 85% (50 W), 79% (40 W), and 77% (50 W with 10% glycerol). Consequently, the use of four different running conditions as opposed to only one increases the sensitivity of the assay by 15% (15 W) to 30% (50 W with 10% glycerol).

DISCUSSION

In this study we have evaluated how different conditions for gel electrophoresis affect analysis of SSCP of the LDLR gene. All gel electrophoreses were run in a cold room to standardize the assay. Running the electrophoreses at room temperature could seriously reduce the reproducibility of the assay as the ambient temperature might exhibit seasonal variations. This is definitely true for most laboratories in Norway.

Regarding the sensitivity of the assay, the seven genotypes analyzed in our pilot study were all correctly identified by analysis of SSCP. This agrees well with the reported data of the sensitivity of SSCP being in the range of 89–100%.⁽³⁾ The higher figure applies to fragments 100–300 bp long, and the lower figure applies to fragments 300–450 bp long. As all our fragments except one are <280 bp, it suggests that the sensitivity of SSCP in detecting point mutations in exons of the LDLR gene must be relatively high. Thus, when four different running conditions are used for the gel electrophoresis, it is plausible that our assumption of a sensitivity of 100% is valid.

Our findings that the overall sensitivity increases by increasing the number of conditions for the gel electrophoresis agrees with those of others.^(1,15) However, employing several running conditions for each PCR fragment is time consuming and must be weighed against the increased sensitivity. The data reported in this paper indicate that using four different running conditions as opposed to only one increases the sensitivity by 15–30%. However, some caution must be exercised in generalizing from these data because they apply in principle only to the specific mutations in our group of patients. Thus, other mutations in the

LDLR gene might require other running conditions to be detected.

It is also important to note that different running conditions should be used for different exons. For genes other than the LDLR gene, where the optimal running conditions for each exon have not been established, our data might indicate that for screening purposes 15 W alone or combined with 50 W will detect the vast majority of the mutations. If the objective of the study is to identify 100% of the mutations, several different conditions should be used. However, in that case, direct DNA sequencing of individual exons might be an alternative strategy.

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