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Fluorescence-based single-strand conformation polymorphism analysis of the low density lipoprotein receptor gene by capillary electrophoresis

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Abstract

We describe here a new method to screen for unknown mutations in the low density lipoprotein (LDL) receptor gene by the use of capillary electrophoresis in single-strand conformation polymorphism (SSCP) analysis. To analyze the promoter and all 18 exons, 20 different amplification reactions were necessary. For each polymerase chain reaction (PCR), the forward and reverse primers were 5′ fluorescent-labelled with FAM and HEX, respectively. To test the accuracy of the newly developed method, 61 genetic variants distributed in 16 exons were analyzed. Under identical electrophoresis conditions (13 kV, 30°C, 30 min), 59 mutations were detected by a distinct abnormal SSCP pattern. The two remaining mutations showed only slight abnormalities, which could be amplified by increasing the electrophoresis temperature. The high accuracy, the degree of automation and the speed of analysis make fluorescence-based SSCP analysis with capillary electrophoresis ideal for rapid mutation screening and the technique is well-suited for clinical applications. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

The low density lipoprotein (LDL) receptor mediates the specific uptake of plasma LDL into cells. Mutations in the LDL receptor gene cause a marked elevation of plasma cholesterol and the clinical phenotype of familial hypercholesterolemia (FH) [1]. In most populations, the heterozygous form of FH is common, affecting approximately one person in 500. Up to now, more than 350 different mutations in the LDL receptor gene have been described [2,3]. Most of these mutations are single base pair substitutions, small insertions or deletions. Although there may exist a preferential location for these mutations in exons 4 and 9, in a diagnostic approach, all 18 exons have to be examined for mutations [4]. Widely used techniques for rapid identification of new mutations are the single strand conformation polymorphism (SSCP) analysis and the denaturing gradient gel

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electrophoresis (DGGE) [5–7]. For several years, we have used SSCP analysis for mutation detection in the LDL receptor gene [8]. The single strands were separated in non-denaturing polyacrylamide gels and, after electrophoresis, the DNA fragments were visualized by silver staining. In our hands, this ''classical'' SSCP analysis in non-denaturing gels has a high accuracy, but only when the running conditions were first optimized for each DNA fragment. For a routine genetic test, this approach is too complicated and time-consuming, especially for the analysis of small DNA sample numbers. To create a more practicable DNA test for a specialized genetic laboratory, a high degree of automation is necessary. The analytical steps that benefit most from automation are the electrophoresis and the visualization of the DNA fragments. Therefore, we explored possibilities for automating this analytical procedure.

Capillary electrophoresis has become widely distributed in clinical laboratories during the last two years. Advantages of these new systems are the injection of new gel after each run and a sample tray in which the DNA samples are placed. These two conditions are necessary to achieve a long walk-away time. The main applications of capillary gel electrophoresis are fragment analysis and sequencing; only a few reports exist regarding the use of capillary electrophoresis in SSCP analysis [9,10]. Because of the extreme heterogeneous localization of the LDL receptor gene mutations and the high frequency of the disease, it made sense to establish a screening test for LDL receptor gene mutations based on a system with a high degree of automation.

The running temperature and the gel matrix in capillary electrophoresis are different from those of conventional SSCP analysis. Therefore, one of the main goals of our investigation was to establish the accuracy of the new method. Sixty-one previously characterized genetic variants (eight polymorphisms and 53 LDL receptor gene mutations) distributed in 16 of the 18 exons were analyzed by fluorescence-based SSCP analysis in combination with capillary electrophoresis. The majority of the mutations studied were point mutations. One other important aim of our work was the standardization of the electrophoresis procedure since running conditions have to be chosen that allow the analysis of all 18 exons.

2. Experimental

2.1. Patients

DNA from 72 individuals with different genetic variations were investigated by fluorescence-based SSCP analysis. Fifty of these individuals had mutations in the LDL receptor gene that causes severe hypercholesterolemia. DNA from these individuals was provided by four centers: the Institute of Clinical Chemistry at the University of Cologne, Germany; Department of Clinical Chemistry, University of Freiburg, Germany; Franz-Volhard-Clinic, Max-Dellbrück-Center for Molecular Medicine, Germany and the Department of Clinical Chemistry, University of Saarlandes, Germany. The mutations were previously characterized by DNA sequencing in these centers. In addition to these well-characterized mutations, DNA samples from 22 healthy individuals were screened for known polymorphisms and their varying genotypes. Whenever available, additional individuals with identical genetic variants were investigated by SSCP analysis.

2.2. Amplification of genomic DNA

The target sequences of the promoter and all 18 exons were amplified by polymerase chain reaction (PCR) in 50 μl of a solution containing approximately 0.5 μg DNA, of 10 pmol of each primer, 250 μmol dNTPs, 1.5 U Taq polymerase (Boehringer, Mannheim, Germany) and 5 μl of buffer, as recommended by the manufacturer of the polymerase. In addition to these standard conditions, the $MgCl_2$ concentration was verified and glycerol was supplemented in some cases (Table 1). All PCR reactions were subjected to 36 cycles at 94°C for 1 min, different annealing temperatures for 45 s (Table 1) and 72°C for 30 s in a RoboCycler Gradient 96 (Stratagene, La Jolla, CA, USA). In each PCR, the upstream and downstream primers were fluorescence-labelled at their 5′ end with FAM or HEX, respectively. The primers of the promoter and of exons 1, 2, 4b, 5, 6, 7, 11, 12, 14–18 were supplied by ABI Perkin Elmer (Weiterstadt, Germany); the remaining primers were supplied by MWG Biotec (Ebersberg, Germany). Because of the length of exon 4 (381 bp), SSCP analysis was performed in

Table 1
Primers for amplification of the human LDL receptor gene[a]

Amplified segment	Fragment size (bp)	Primer sequence (5′ to 3′) Sense primer	Antisense primer	Special comment[b]
Promoter	277	CAGCTCTTCACCGGAGACC	ACCTGCTGTGTCCTAGCTGG	(58/3/1)
Exon 1	223	TGAAATGCTGTAAATGACGTGG	GCTCCCTCTCAACCTATTCTGG	(56/3/−)
Exon 2	198	GTTTCTGATTCTGGCGTTGAG	CATATCATGCCCAAAGGGG	(54/4/−)
Exon 3	196	TTCCTTTGAGTGACAGTTCAATCC	GATAGGCTCAATAGCAAAGGCAGG	(56/4/−)
Exon 4a	242	GTGGTCTCGGCCATCCATCC	AGCCATCTTCGCAGTCGGGG	(60/3/−)
Exon 4b	236	CGACTGCGAAGATGGCTCGGA	GGGA_CCCAGGGACAGGTGATAGGAC	(58/4/1)
Exon 5	239	AAGGCCCTGCTTGTTTTTCT	TGCTTGGCAGAGAATGGG	(54/4/−)
Exon 6	216	CTGACCTTCCTCCTTCCTCTCT	AACCTCCACCTTTCCTGGCT	(58/4/−)
Exon 7	211	GGCCCGAGAGTGACCAGT	CATGTCAGGAAGCGCAGAG	(56/4/1)
Exon 8	220	CATTGGGGAAGAGCCTCCCC	GCCTGCAAGGGGTGAGGCCG	(60/3/1)
Exon 9	224	CCCCTGACCTCGCTCCCCGG	GCTGCAGGCAGGGGCGACGC	(60/3/1)
Exon 10	278	ATGCCCTTCTCTCCTCCTGC	AGCCCTCAGCGTCGTGGATA	(60/4/1)
Exon 11	179	GCCTCACAGCTATTCTCTGTCCTCC	TCCCTGTGACGGCTGTCCTGCGAAC	(58/2/1)
Exon 12	210	GCACGTGACCTCTCCTTATCCACTT	CACCTAAGTGCTTCGATCTCGTACG	(58/3/−)
Exon 13	216	GTCATCTTCCTTGCTGCCTG	TTCCACAAGGAGGTTTCAAGGTTG	(58/4/−)
Exon 14	228	TGCCCTGACTCCGCTTCT	GGGGCAGTTGGAGGACAC	(56/4/1)
Exon 15	221	AGAAGACGTTTATTTATTCTTTC	GTGTGGTGGCGGGCCCAGTCTTT	(50/6/1)
Exon 16	202	CCTGCTCCATTTCTTGGTGG	ACGAGGTCACATAGCGGGAG	(56/3/−)
Exon 17	246	AGCTGGGTCTCTGGTCTCGGAGGC	GGCTCTGGCTTTCTAGAGAGGG	(58/3/1)
Exon 18	190	TCCAGCCTGTTTCCTGAGTGCTGG	CAGGCAATGCTTTGGTCTTCTCTG	(56/3/−)

[a] The primer sequences underlined are nucleotide substitutions as a consequence of primer optimization. In the antisense primer of exon 4b, a G is deleted in the underlined position.

[b] Under "Special comment", the PCR conditions are encoded. The first position in the code is the annealing temperature, the second position is the volume (μl) of added $MgCl_2$ (25 mM) and the third position indicates whether glycerol (870 ml/l) was added.

two steps (exons 4a and 4b) [11]. The promoter and all other exons were analyzed in one step. With the exception of the internal primers of exon 4, the primers were localized in the neighboring introns. For some of the exons, the primer sequences have already been reported (Jensen et al. [12]: Promoter, exon 3, 4a, 8, 9, 10 and 15; Hobbs et al. [13]: Exon 12; Gudnason et al. [14]: Exon 4b). After amplification, agarose gel electrophoresis was performed as a control on the PCR product.

2.3. SSCP analysis

Capillary electrophoresis was performed with the ABI 310 genetic analyzer (PE Applied Biosystems, Foster City, CA, USA) in a 50 g/l GeneScan polymer (PE Applied Biosystems) with 100 ml/l glycerol and 1×Tris–borate–EDTA buffer (0.1 mol/l Tris, 0.077 mol/l boric acid and 0.0025 mol/l EDTA, pH 8.5). For capillary electrophoresis, the PCR product was diluted 5–30 fold with water and, to 1 μl of the diluted PCR product, were added 10.5 μl of formamide, 0.5 μl of size standard (GeneScan-500 Tamra, PE Applied Biosystems) and 0.5 μl of 0.3 mol/l NaOH. The SSCP mix was denatured at 90°C for 2 min, chilled on ice and finally placed in the tray of the analyzer. Electrophoretic analysis was performed in a 47-cm capillary with an I.D. of 50 μm (PE Applied Biosystems) at 13 kV for 30 min. The length of the capillary to the detector was 36 cm. The buffer consisted of a 1×Tris–borate–EDTA buffer with 100 ml/l glycerol. After each run, the used polymer solution was pressed out of the capillary and automatically replaced by a fresh one. The detection system consisted of an argon ion laser that activated the fluorescent-labelled DNA fragments at 488 and 515 nm. The emitted light was measured for FAM at 535 nm, for HEX at 559 nm and for TAMRA at 580 nm. To eliminate run-to-run variations, the migration times of the single strand peaks were standardized to the internal size marker. The SSCP analyses were performed at different running temperatures (30, 35, 40 and 45°C), which was achieved using a thermostable plate. All procedures

were controlled by parameter input to the software system and were completely automated.

2.4. Multiplex analysis

For each exon, both of the single strands have characteristic migration times in capillary electrophoresis. To analyze the whole LDL receptor gene, 20 different amplification products were necessary. Depending of the migration time of the single strands, combinations of PCR products were chosen where the single strand peaks labelled with the same fluorescence dye did not overlap. To minimize the complexity of the peak pattern, the multiplex analysis was limited to the combination of two PCR products. The exons were amplified in a multiplex PCR or the PCR products were mixed after the amplification step. In our analysis, we preferred the mixture of the DNA samples after amplification, since the advantage of this procedure is the possibility of diluting the individual PCR products with water according to the calculated quantity in the agarose gel. Then the PCR products were mixed and analyzed in a manner similar to that described above.

3. Results and discussion

In our approach, SSCP analysis was adapted to capillary electrophoresis with fluorescence detection for mutation screening of the LDL receptor gene. The combination of fluorescence-based SSCP analysis with capillary electrophoresis offers, for the first time, a high degree of automation. In the ABI 310 genetic analyzer, all electrophoresis procedures, including the replacement of the gel, are controlled by parameter input to the software system. The LDL receptor gene consists of 18 exons. The number and length of the exons of the LDL receptor gene resulted in 20 different fragments, which are necessary to analyze the whole gene. For the separation of the single strands, a GeneScan™ polymer with 100 ml/l glycerol was used. The primers used for amplification were 5′-labelled with a fluorescent dye. To differentiate between the sense and antisense single strands, the primers were labelled with different fluorescent dyes. The upstream primer was labelled with FAM and the downstream primer with HEX, which had advantages in peak evaluation because, even in cases where an overlap between the two single strands existed, an unambiguous interpretation of the peak pattern was possible. The primer sequences used are presented in Table 1. These primers were chosen for two reasons. The primers should be localized outside the splicing sites of the exons, and the length of the fragments studied should be less than 300 bp. With increasing length of the PCR fragments, a reduced ability to detect mutations was observed. For example, from five mutations of exon 4 analyzed in one step (fragment length, 462 bp), only three could be detected. To optimize the primer, we introduced single nucleotide substitutions into the original sequence in some cases. The exchanged nucleotides are underlined in the table. Fig. 1 shows the results of SSCP analysis of exon 4b. The complementary single strands were completely separated and each single strand is demonstrated as a single peak (Fig. 1a). The sense strand, labelled with FAM, is presented as a solid line; the line of the antisense strand, labelled with HEX, is dotted. In Fig. 1b and 1c, additional peaks indicates mutations (Fig. 1b: A→G single nucleotide substitution at position 662 and Fig. 1c: combined 5 bp deletion/6 bp insertion mutation). However, in not every case was the single strand of the wild-type allele detected as the expected single peak; in some cases, a complex peak pattern, as shown for the C→T transition in exon 13, was found (Fig. 2a, homozygous C; Fig. 2b, heterozygous and Fig. 2c, homozygous T). The explanation for the additional peaks was most likely additional stabilized states of the single strands. More important than the appearance of the peak pattern is the reproducibility of the SSCP analysis. The high reproducibility was achieved by a size marker, GenScan-500 labelled with the fluorescence dye TAMRA, which was added to each PCR product. The peaks of the size marker were defined by their data point localization. The data points of the single strand peaks of the LDL receptor gene were evaluated by the software in relation to the peaks of the size marker. For the two exons shown in Figs. 1 and 2 and for all other fragments analyzed, excellent reproducibility of the SSCP peak pattern was found. In relation to the size marker, a mean standard deviation of 2.9 data points for 46 evaluated peaks was achieved in day-to-day electrophoresis

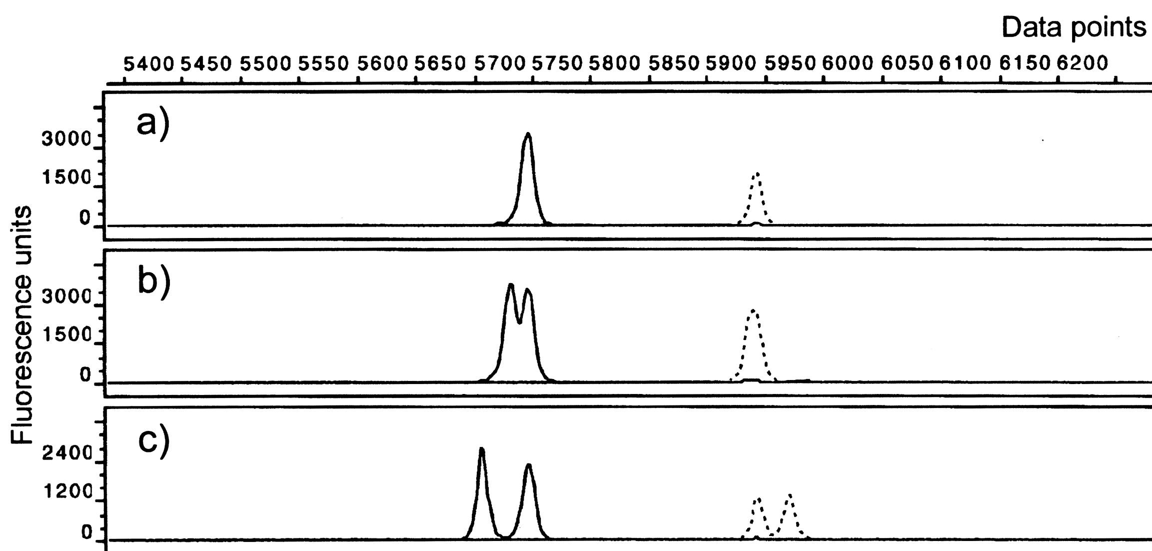

Fig. 1. Electropherograms representing fluorescence-based SSCP analysis of exon 4b of the LDL receptor gene by capillary electrophoresis. The two complementary single strands are labelled with different fluorescent dyes (sense strand with FAM and the antisense strand with HEX). To differentiate the two single strand signals in an uncoloured figure, the FAM- and the HEX-labelled single strands are presented as solid and dotted lines, respectively. In (a), the normal SSCP pattern is presented; in (b) and (c), additional peaks indicate mutations; (b) nucleotide position 662, A/G; (c) heterozygous 5 bp deletion/6 bp insertion mutation.

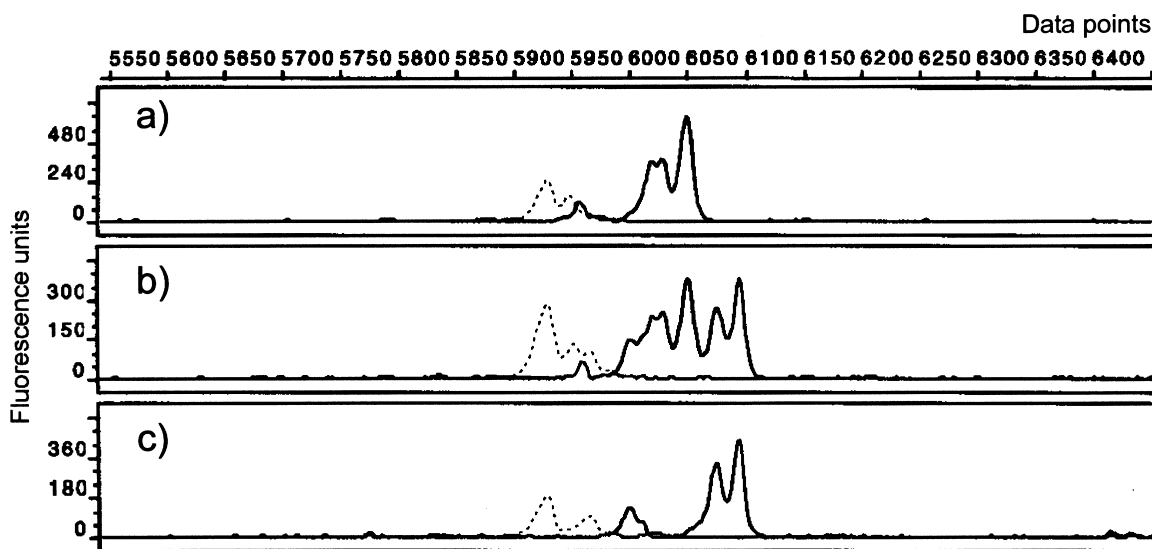

Fig. 2. Electropherograms representing fluorescence-based SSCP analysis of exon 13 of the LDL receptor gene by capillary electrophoresis. The differentiation of the sense and antisense strand signals are as in Fig. 1. The three genotypes of the AvaII polymorphism (nucleotide position 1959) in exon 13 are presented. (a) C/C; (b) C/T; (c) T/T.

runs. The reproducibility, even in cases of a complex peak pattern, is the prerequisite for detection of abnormal peak patterns indicating mutations.

In classical SSCP analysis with polyacrylamide gels, one of the factors that has distinct influence on the peak pattern and on mutation detection is the temperature during electrophoresis. To verify the effect of temperature in capillary electrophoresis, we performed SSCP analysis at different temperatures. The exons 3, 4a, 8, 9, 10 and 13 were systematically tested at 30, 35, 40 and 45°C. In summary, the mutations described in these exons (Table 1) were clearly detected at 30, 35 and 40°C, but not in all cases at 45°C. From these first three temperatures, a trend towards the best separation of the complementary single strands and mutation detection was found at 30°C. At 40 and 45°C, the distance between the complementary single strands diminishes, which makes the identification of mutations in some cases more difficult. Therefore, in our ongoing experiments we preferred the 30°C temperature for studying all exons.

From these initial experiments, capillary electrophoresis is suitable in principal for SSCP analysis, but questions as to the accuracy of the method have to be answered. To get an impression of the accuracy, known genetic variants in the LDL receptor gene were studied. Eight of these variants were polymorphisms already described in the literature; the remaining 53 genetic variants were mutations causing hypercholesterolemia. The polymorphisms were first characterized by restriction enzyme digestion or sequencing; the mutations detected in hypercholesterolemic individuals were already verified by sequencing before starting the capillary electrophoresis. The genetic variants showed a wide range of different mutations. Forty-eight of these variants were single nucleotide substitutions, six were small deletions (2×1 bp, 3 bp, 4 bp, 7 bp 37 bp), six were small insertions (2×1 bp, 2 bp, 4 bp, 8 bp, 11 bp) and one was a combined insertion/deletion mutation (5 bp ins, 6 bp deleted) (Table 2). Genetic variants were available for all exons with the exception of the promoter, exon 16 and 17. The distribution of mutations in a large number of exons was necessary to calculate whether or not the fluorescense-based SSCP analysis in combination with capillary electrophoresis is suitable for detecting unknown mutations in a particular exon. From the 61 different mutations studied, a distinctly abnormal SSCP pattern was found in 59 cases, indicating that genetic variants were present. The high rate of mutation detection (96%) indicates that the capability of capillary electrophoresis in SSCP analysis is comparable with, or even higher than, classical SSCP analysis in polyacrylamide gels.

The two mutations that showed only slight abnormalities in SSCP patterns were analyzed in more detail. To our surprise, one of these two mutations was not a single nucleotide substitution, but a one base-pair insertion. For the 1 bp insertion, the abnormalities in SSCP pattern were a less pointed peak and a decreased height of the antisense strand in relation to the sense strand. This abnormality was reproducible from run to run. By increasing the electrophoresis temperature to 45°C, the mutation could be clearly recognized by a distinctly abnormal SSCP pattern. The insertion was a cytosine in a region of five cytosines. Obviously, this combination of nucleotides has such a stable single strand conformation that the 1 bp insertion results in no conformational change. In this special case, the abnormally high electrophoresis temperature is obviously necessary to provoke the destabilization of the single strand, which then results in an abnormal SSCP pattern. The second mutation, a C to T transition, was detected at 30°C only by a slightly rounder curve at the top of the peak. This abnormality was also reproducible from run to run. The reason why this mutation was so difficult to detect remains unclear. A large number of the other single nucleotide substitutions were also transitions and a C to T exchange was also present. By increasing the temperature, the abnormal SSCP pattern became more prominent, but at temperatures up to 45°C, there were no dramatic changes. The distinct SSCP pattern indicating the mutation was finally found at 48°C.

In addition to the index subjects, each with a characteristic SSCP pattern, subjects with identical polymorphisms or mutations were analyzed. In each case, the genetic variant could be unambiguously identified by the characteristic SSCP pattern. A great advantage of this system is the possibility of data storage. The data on the SSCP pattern can be stored in the software which is the basis for creating a data catalogue for different mutations. If a mutation is

Table 2
Characterization of genetic variations in the LDL receptor gene by SSCP analysis and capillary electrophoresis

Location	(Literature/DNA)[a]	Nucleotide change	Location	(Literature/DNA)[a]	Nucleotide change
Exon 1, nt 1	([15]/c)	ATG→GTG	Exon 6, nt 917	([2,3]/b)	TCA→TTA
Exon 2, nt 81	([3]/d)	TGT→TGC[b]	Exon 7, nt 970	([2,3]/b)	1 bp del
Exon 3, nt 196	(x/a)	2 bp ins AT	Exon 7, nt 1013	(x/c)	TGC→TAC
Exon 3, nt 224	([2,3]/a)	TGT→TAT	Exon 8, nt 1171	([3]/a, d)	GCC→ACC[b]
Exon 3, nt 253	([2,3]/b)	CAG→TAG	Exon 9, nt 1285	([2,3]/a, b)	GTG→ATG
Exon 3, nt 259	([2,3]/a, c)	TGG→GGG	Exon 9, nt 1307	([2,3]/a)	GTG→GCG
Exon 3, nt 301	([2,3]/a)	GAG→AAG	Exon 9, nt 1329	([2,3]/a)	TGG→TGC
Intron 3, 313+2	([3]/c)	T→C	Exon 9, nt 1338	(x/a)	CTG→CTT
Exon 4a, nt 315	(x/a)	1 bp ins	Exon 9, nt 1340	(x/a)	TCC→TGC
Exon 4a, nt 324	(x,/a)	ACG→ACT	Exon 10, nt 1413	([3]/a, d)	AGG→AGA[b]
and nt 325	[2,3]	TGC→CGC	Exon 10, nt 1426	(x/a)	4 bp ins
Exon 4a, nt 400	(x/a)	TGC→CGC	Exon 10, nt 1429	(x/a)	1 bp ins
Exon 4a, nt 418	(x/c)	GAG→GAC	Exon 10, nt 1444	([2,3]/a)	GAC→AAC
Exon 4a, nt 419	(x/a)	GAG→GGG	Exon 10, nt 1567	([2,3]/a)	GTG→ATG
Exon 4a, nt 500	([2,3]/b)	TGC→TAC	Exon 10, nt 1570	(x/a)	GTG→TTG
Exon 4a, nt 501	([2,3]/a)	TGC→TGA	Exon 11, nt 1646	([2,3]/d)	CGC→TGC
Exon 4b, nt 517	([2,3]/b)	TGC→GGC	Exon 12, nt 1720	(x/c)	CGC→TGC
Exon 4b, nt 518	([2,3]/a)	TGC→TAC	Exon 12, nt 1725	([3]/a, d)	CTC→CTT[b]
Exon 4b, nt 519	([2]/a, c)	TGC→TGG	Exon 12, nt 1773	([3]/a, d)	AAT→AAC[b]
Exon 4b, nt 530	(x/b)	TCG→TTG	Exon 12, nt 1784	([2,3]/b)	7 bp del
Exon 4b, nt 531	([2,3]/a)	8 bp ins	Exon 13, nt 1873	(x/c)	AAC→ACC
Exon 4b, nt 615	(x/c)	11 bp ins	Exon 13, nt 1920	(x/d)	AAC→AAT
Exon 4b, nt 651	([2,3]/a)	37 bp del	Exon 13, nt 1959	([3]/d)	GTC→GTT[b]
Exon 4b, nt 652	([2,3]/a)	1 bp del	Exon 14, nt 2042	([2,3]/b)	TGC→TAC
Exon 4b, nt 652	([2,3]/a)	3 bp del	Exon 14, nt 2054	([2,3]/c)	CCG→CTG
Exon 4b, nt 656	([2]/a)	5 bp del+6 bp ins	Exon 14, nt 2096	([2,3]/b)	CCG→CTG
Exon 4b, nt 662	([2,3]/a)	GAC→GGC	Exon 15, nt 2167	(x/d)	1 bp del G
Exon 4b, nt 671	([2,3]/a)	GAC→GTC	Exon 15, nt 2177	([2,3]/c)	ACC→ATC
Exon 4b, nt 682	([2,3]/a, c)	GAG→TAG	Exon 15, nt 2225	(x/c)	ACC→ATC
Exon 5, nt 757	([2]/c)	CGG→TGG	Exon 15, nt 2232	([3]/a, d)	CGG→CGA[b]
Exon 5, nt 798	([2,3]/b)	GAT→GAA	Exon 18, nt 2635	([3]/a, d)	GGC→AGC[b]

[a] In the column (Literature/DNA) the first position in the bracket refers to the databases where the mutations are listed (see references), x describes a not previously published mutation. The second position refers to the center where the DNA was received (a, Institute of Clinical Chemistry, University of Cologne, Germany; b, Franz-Volhard-Clinic, Max-Dellbrück-Center for Molecular Medicine, Germany; c, Department of Clinical Chemistry, University of Freiburg, Germany and d, Department of Clinical Chemistry, University of Saarland, Germany).

[b] These genetic variants are polymorphisms described in the literature.

detected in a diagnostic approach, the SSCP pattern can be extended to similar patterns stored in the database. If there is an agreement between the SSCP pattern of a newly identified mutation and a stored pattern, the kind of mutation is nearly characterized. This simplifies the sequencing step that follows because the evaluation can be focused on a small region of interest.

The capillary electrophoresis system used is a completely automated system with a sample tray. One SSCP analysis with time for replacement of the gel takes 36 min. Since 20 fragments have to be analyzed, the screening of the whole LDL receptor gene requires approximately 12 h. To shorten the analysis time per individual, multiplex analysis was performed. In creating multiplex analysis steps, it is important that there exist no overlaps between the single strands with the same fluorescent-dye label. In view of the migration time and fluorescence labelling of the single strands, the following combinations were performed as multiplex analysis: Promotor+ exon 18, exon 7+11, exon 2+17, exon 3+12, exon 8+5, exon 9+10 and exon 13+16. With the two available fluorescent dyes, a combined analysis is

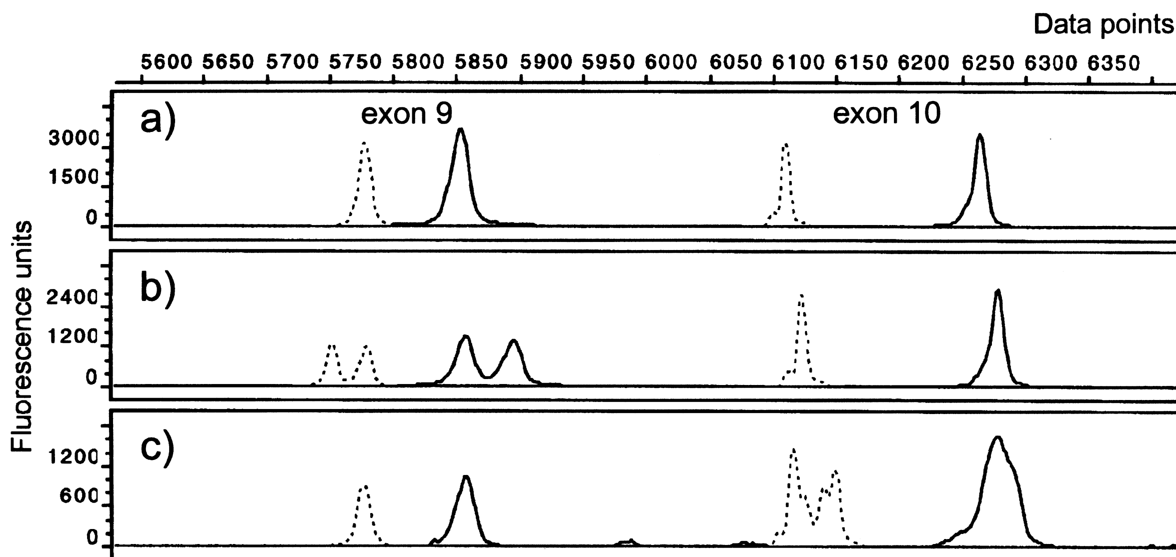

Fig. 3. Electropherograms representing multiplex analyis of exons 9 and 10 of the LDL receptor gene by capillary electrophoresis. The differentiation of the sense and antisense strand signals are as in Fig. 1. Three single nucleotide substitutions, one in exon 9 (nucleotide position 1307) and two in exon 10 (nucleotide positions 1413 and 1567), were analyzed in different combinations. The PCR products were joined after dilution. The following analysis was performed in an identical manner to that for single samples. (a) exon 9, T/T; exon 10, nt 1413 G/G and nt 1567 G/G; (b) exon 9, T/C; exon 10, nt 1413 G/G and nt 1567 G/G; (c) exon 9, T/T; exon 10, nt 1413 G/A and nt 1567, G/A.

limited to two fragments at the moment. In these fragment combinations, the mutations described in Table 2 were analyzed. One example of a multiplex analysis is presented in Fig. 3. Three different combinations of three single nucleotide substitutions, one in exon 9 (nt 1307) and the two others in exon 10 (nt 1413 and nt 1567), were analyzed (Fig. 3a: exon 9, T/T; exon 10, nt 1413 G/G and nt 1567 G/G; Fig. 3b: exon 9, T/C; exon 10, nt 1413 G/G and nt 1567 G/G; Fig. 3c: exon 9, T/T; exon 10, nt 1413 G/A and nt 1567, G/A). By using multiplex analysis, the whole gene could be analyzed in 7.8 h without compromising the quality. In the future, if there are additional fluorescent dyes available that allow parallel applications, more than two exons could be analyzed in one step.

4. Conclusions

In summary, familial hypercholesterolemia is a relatively common genetic disease. In the majority of cases, the mutations are single nucleotide substitutions, small insertions and deletions heterogeneously localized in the gene. If the diagnosis of familial hypercholesterolemia is to be performed on a genetic level, mutation screening procedures with a high degree of automation are necessary. Fluorescence-based SSCP analysis in combination with capillary electrophoresis offers, for the first time, an approach that fulfils this demand. Besides the high accuracy in mutation detection, the data management by the software permits definition and archiving of a characteristic SSCP pattern for each mutation.

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