papers and notes on methodology

Mutations in the low density lipoprotein receptor gene of familial hypercholesterolemic patients detected by denaturing gradient gel electrophoresis and direct sequencing

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Abstract Familial hypercholesterolemia (FH) results from mutations in the low density lipoprotein receptor (LDLR) gene. We applied denaturing gradient gel electrophoresis (DGGE) to screen for sequence variations in the coding and splice site consensus sequences of the LDLR gene. For amplification of each exon by the polymerase chain reaction (PCR), optimal pairs of primers were designed by the MELT 87 computer algorithm. To increase the sensitivity, an artificial GC-clamp was included in either the 5'- or the 3'-end of each fragment. DGGE screening of 32 apparently unrelated heterozygous FH patients revealed 16 unique different aberrant DGGE patterns in 27 patients, while in a group of 32 normal subjects none of these DGGE patterns could be observed, suggesting that the aberrant patterns represent disease-causing mutations. Interestingly, 16 out of 27 patients showed an aberrant DGGE pattern in the part of the gene encoding the ligand binding domain (exons 2–6). Direct solid-phase sequencing of the corresponding exon-specific PCR products revealed the nature of the mutations: three nonsense, four splicing, two frameshift, one silent, and six missense mutations. Six of the mutations have been previously reported, while ten are novel mutations. These results indicate that DGGE provides a reliable method for the detection of the presence of point mutations in the LDLR gene of FH patients, thereby facilitating the introduction of rapid DNA diagnosis for this common and genetically heterogeneous disorder.—**Lombardi, P., E. J. G. Sijbrands, K. van de Giessen, A. H. M. Smelt, J. J. P. Kastelein, R. R. Frants, and L. M. Havekes.** Mutations in the low density lipoprotein receptor gene of familial hypercholesterolemic patients detected by denaturing gradient gel electrophoresis and direct sequencing. *J. Lipid Res.* 1995. **36:** 860–867.

Supplementary key words low density lipoprotein receptor • mutations • polymerase chain reaction

Familial hypercholesterolemia (FH) is an autosomal, dominantly inherited disease caused by mutations in the gene encoding the low density lipoprotein receptor (LDLR), which mediates the specific uptake of plasma LDL. In most populations, the heterozygous form of FH is common, affecting approximately one person in every 500. Heterozygous FH individuals have only half the normal number of functional LDL receptors and, as a consequence, plasma LDL cholesterol levels are about twice the normal level, leading to premature atherosclerosis. Homozygous FH patients are more severely affected and rarely reach the age of maturity (1). Homozygous FH is rare and clinically unmistakable, while it may be difficult to make an unequivocal diagnosis of heterozygous FH, unless the presence of tendon xanthomas or a well-defined family history of hypercholesterolemia has been recorded.

After the LDL receptor gene was cloned (2), more than 150 mutations including insertions, deletions, nonsense and missense mutations were described, affecting either the synthesis, post-translational processing, ligand binding activity, or internalization of the LDL receptor (3). Most mutations so far characterized appeared to be unique, except for those cases, in some isolated populations, where a founder effect has resulted in allelic enrichment (4–7). Approximately one-third of the mutations consists of major structural rearrangements detectable by Southern blotting (3, 8–10). The remaining mutations are predominantly point mutations or small insertions or deletions (3). This marked genetic heterogeneity of FH has so far precluded the development of simple screening tests for the identification of mutations in the LDL receptor

Abbreviations: FH, familial hypercholesterolemia; LDLR, low density lipoprotein receptor; PCR, polymerase chain reaction; DGGE, denaturing gradient gel electrophoresis.

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gene in individual patients. Such tests may facilitate studies on the genotype–phenotype relationship and the effect of treatment.

In recent years, several reports have shown that denaturing gradient gel electrophoresis (DGGE) is a powerful method for the near complete detection of the presence of disease-related point mutations and polymorphisms (11–13). In the present study, we report the development of a GC-clamped DGGE assay that enables an efficient screening of sequence variations of the coding and splice-site consensus sequences of the LDL receptor gene. In a group of 32 apparently unrelated heterozygous FH patients, we identified the previously reported common polymorphisms (14–20) and 28 sequence alterations in 27 patients. None of these alterations has been found in normal subjects and represent, in total, 16 different candidate FH-causing mutations. Sequence analysis revealed that, among these, 6 are known mutations (3) whereas 10 are novel mutations.

MATERIALS AND METHODS

Subjects

Thirty-two apparently unrelated patients with a clinical diagnosis of heterozygous FH were recruited from the Lipid Clinic of the Leiden University Hospital. The selection criteria were: *i)* mean fasting total serum cholesterol concentration ≥ 9.5 mmol/l after 9 weeks of eucaloric diet and without the use of lipid lowering drugs; *ii)* mean fasting triglycerides < 2 mmol/l; *iii)* presence of tendon xanthomas; or *iv)* occurrence of hypercholesterolemia or early onset of coronary artery disease in first degree relatives; *v)* absence of structural rearrangements in the LDL receptor gene by conventional Southern analysis performed as previously described (10); and *vi)* absence of the B3500 mutation, characteristic of familial defective apolipoprotein B (FDB) (21).

Control individuals were healthy normolipidemic volunteers from the RIFOH study (22).

Melting map predictions

Melting maps of each genomic (exon) fragment with a GC-clamp attached to either the 5'- or the 3'-primer were generated with the MELT87 computer algorithm (23). MELT87 calculates the melting temperature (T_m) of a given DNA sequence as a function of its nucleotide sequence and composition.

DNA isolation

Genomic DNA was isolated from peripheral leukocytes, according to the method of Miller, Dykes, and Polesky (24).

Amplification of the exons of the LDL receptor gene by the polymerase chain reaction (PCR)

PCR primers, homologous to the intron sequences flanking the 18 exons of the LDL receptor gene, were designed on the basis of the sequences published by Leitersdorf et al. (6). Primers were synthesized on a Bioresearch Cyclone™ synthesizer. A list of primer sequences is given in **Table 1.** To optimize the resolving power of DGGE, a two-step PCR protocol was developed to obtain amplification products with an artificial high-

TABLE 1. Oligonucleotide primers for PCR amplification of individual exons of the human LDL receptor gene

Exon	5'-Primer (5'→3')		3'-Primer (5'→3')	
1	B[a] 2772[b]	15bpGC-AATGCTGTAAATGACGTGG[c]	A 2773	TTCTGGCGCCTGGAGCAAG
2	B 3118	15bpGC-CCTTTCTCCTTTTCCTCTCTCTC	A 3119	AAAATAAATGCATATCATGCCCA
3	B 3078	15bpGC-TGACAGTTCAATCCTGTCTCTTC	A 3079	AATAGCAAAGGCAGGGCCACACT
4	A 2534	TGGTCTCGGCCATCCATCC	B 2535	15bpGC-ACGCCCCGCCCCCACCCTG
5	A 2536	CAACACACTCTGTCCTGTT	B 2537	15bpGC-GGGAAAACCAGATGGCCAGC
6	B 2668	15bpGC-TCCTTCCTCTCTCTG	A 2669	GCAAGCCGCCTGCACCGAG
7	B 3120	15bpGC-AGTCTGCATCCCTGGCCCTGCGC	A 3121	AGGGCTCAGTCCACCGGGGAATC
8	B 2467	15bpGC-CCAAGCCTCTTTCTC	A 2468	CCACCCGCCGCCTTCCCGT
9	B 1205	15bpGC-GGCTGCAGGCAGGGGCGACG	A 1289	CTGACCTCGCTCCCCGGACC
10	A 2715	ATGCCCTTCTCTCCTCCTG	B 2716	15bpGC-AGCCCTCAGCGTCGTGGAT
11	B 2713	15bpGC-CAGCTATTCTCTGTC	A 2714	TGGCTGGGACGGCTGTCCT
12	B 2469	15bpGC-TCTCCTTATCCACTT	A 2470	TTCGATCTCGTACGTAAG
13	B 3122	15bpGC-GTCATCTTCCTTGCTGCCTGTTT	A 3123	GTTTCCACAAGGAGGTTTCAAGG
14	B 2717	15bpGC-CCTGACTCCGCTTCT	A 2718	ACGCAGAAACAAGGCGTGT
15	A 3124	AGAAGACGTTTATTTATTCTTTC	B 3125	15bpGC-GTGTGGTGGCGGGCCCAGTCTTT
16	B 2770	15bpGC-CCTCACTCTTGCTTC	A 2771	CGCTGGGGGACCGGCCCGC
17	B 2768	15bpGC-TGACAGAGCGTGCCTC	A 2769	TGGCTTTCTAGAGAGGGTC
18	B 2774	15bpGC-TCCGCTGTTTACCATT	A 2775	TCTCAGGAAGGGTTCTGGG
GC clamp	C 2548	CGCCCGCCGCGCGCCGCGCCCGTCCCGCCGCCGCC-CGCCGCCGCCGCCCG		

[a] Designation.
[b] Code.
[c] The underline represents the 15 bp GC-rich sequence that acts as a linker.

melting domain (GC-clamp) (25, 26). One primer of each set (primer B) contained at the 5'-end a GC-sequence of 15 nucleotides that acts as a linker. The primer that did not contain the GC-rich sequence is indicated as A. A biotin group was attached to primers A during synthesis to allow subsequent solid-phase direct sequencing of the fragments. The 15 bp GC-rich sequence was further elongated to a 50 bp GC-rich sequence in a second PCR run, using primer C, a 50-mer that had, at its 3'-end, a sequence identical to the GC-stretch in primer B. The remaining 35 bp of this primer were random Gs and Cs. In general, PCR was carried out on approximately 500 ng of genomic DNA in a buffer containing 10 mM Tris-HCl, pH 8.4, 50 mM KCl, 0.2 mM of each deoxy nucleoside 5'-triphosphate (dNTP), 50 pmol of each primer, and 1 unit of *Taq* DNA polymerase (Amplitaq, Cetus, Norwalk, Conn.).

Experimental details, including DMSO and $MgCl_2$ concentrations, are given in **Table 2.** The amplification protocol consisted of 1 min denaturation at 94°C, 30 sec annealing at 55°C and 90 sec extension at 72°C for 32 cycles. Slightly different amplification protocols were required for exon 12 (annealing temperature of 47°C) and for exon 9 (1 min at 94°C, 90 sec at 62°C, and 2 min at 72°C). The same PCR amplification protocols were used in the second PCR round to obtain the AC product with the following modification: the PCR mixture contained 1 μl of the AB product, 40 pmol of primer A, and 20 pmol of primer C (50 nucleotide universal GC-rich primer).

TABLE 2. PCR conditions for amplification of the individual exons of the LDLR gene

Exon	DMSO[a] (10% v/v)	$MgCl_2$	Denaturing Gradient
		mM	%
1	–	1.5	60–80
2	+	1.5	40–75
3	+	1.5	40–75
4	+	1.0	50–80
5	+	1.5	50–75
6	+	1.5	40–70
7	–	1.0	50–80
8	–	1.0	50–80
9	+	1.0	50–70
10	+	1.5	45–75
11	–	1.5	35–65
12	–	1.5	35–75
13	–	1.5	35–70
14	–	1.5	40–70
15	–	1.5	40–80
16	–	1.5	55–80
17	–	1.5	35–65
18	–	1.5	50–80

[a] (+) and (–) indicate the presence or absence of DMSO (10% final concentration).
[b] One hundred percent denaturant is equivalent to 7 M urea and 40% (v/v) deionized formamide.

The abundance and quality of the DNA fragments were analyzed by electrophoresis on 2% agarose gels, followed by ethidium bromide staining and inspection under UV light.

Denaturing gradient gel electrophoresis (DGGE)

DGGE was carried out according to Fischer and Lerman (27), as modified by Top et al. (28). GC-clamped amplified DNA fragments were run on 9% polyacrylamide gels containing linearly increasing gradients of denaturant (Table 2). Denaturing gradients were prepared from two stock solutions: *i)* 9% acrylamide without denaturant (0%), and *ii)* 9% acrylamide with 80% denaturant (100% denaturant is equivalent with 7 M urea and 40% (v/v) deionized formamide). The DGGE gradient for each exon was designed according to the melting temperature predicted by the melting maps; the optimal conditions, summarized in Table 2, were obtained when the DGGE bands appeared in the middle of the gel. Electrophoresis was performed at 80 V (6 V • cm^{-1}) for 20 h at constant 60°C in TAE buffer (40 mM Tris-acetate, pH 7.5, 1 mM EDTA). After electrophoresis, the gels were stained in ethidium bromide and examined under UV illumination.

Sequencing of PCR fragments

The exon-specific fragments containing a sequence variation were amplified by PCR. For solid-phase direct sequencing, single-stranded DNA fragments were generated with Dynabeads M-280 Streptavidin (Dynal, Oslo, Norway). Sequence reactions were performed according to the Sequenase version 2.0 protocol (USB). The same pairs of primers were used for amplification and sequencing. The sequencing reactions were electrophoresed on 6% polyacrylamide gels containing 7 M urea, and the results were visualized by autoradiography.

RESULTS

Calculation of the melting maps

For amplification of each exon and flanking exon–intron junctions, primers were designed that were complementary to the intron sequences (6). To increase the sensitivity of DGGE, a 50-base pair GC-clamp was included in either the 5'- or the 3'-end of each amplified fragment by a two-step PCR amplification protocol (26). For each exon, melting maps (23) of genomic fragments containing the GC-clamp either on the 5'-end or on the 3'-end were compared. In each case, the GC-clamp attachment site that predicted the most uniform low-melting domain for the genomic sequence was chosen. The calculated melting maps indicate that in each one of the 18 amplified exons, the entire exon and its 5'- and 3'-flanking intron sequences are contained in a uniformly low melting domain (not shown), thus predicting a high efficiency in screening for DNA sequence variations.

TABLE 3. Polymorphisms

Exon	DGGE Designation	Genotype: FH Group (n = 32)[a] 1.1[b]	1.2[b]	2.2[b]	Control Group (n = 32)[a] 1.1[b]	1.2[b]	2.2[b]
2	2b	0	6	26[c]	0	6	26
8	8a	0	3	29[c,d]	0	2	30[c]
10	10c	10	20	2	10	17	5
11	11b	28	3	1	29	3	0
12	12b	8	14	10[d]	15	12	5
13	13a	10	15	7[d]	11	18	3
15	15b	19	13	0	20	10	2

[a] N represents the number of subjects included in each group.
[b] One and 2 indicate the absence or the presence of the sequence variation, respectively, as compared to the LDLR sequence published by Yamamoto et al. (29).
[c] Presence of the sequence variation is more frequent than the absence (14, 15).
[d] Data representing the results of both DGGE and conventional RLFP analysis.

TABLE 4. Candidate mutations

Exon	DGGE Designation	Genotype: FH Group (n = 32)[a] 1.1[b]	1.2[b]	2.2[b]	Control Group (n = 32)[a] 1.1[b]	1.2[b]	2.2[b]
2	2a	30	2	0	32	0	0
3	3a	31	1	0	32	0	0
	3b	29	3	0	32	0	0
	3c	31	1	0	32	0	0
4	4a	29	3	0	32	0	0
	4b	31	1	0	32	0	0
	4c	31	1	0	32	0	0
6	6a	30	2	0	32	0	0
	6b	30	2	0	32	0	0
7	7a	29	3	0	n.t.[c]	n.t.	n.t.
9	9a	31	1	0	n.t.	n.t.	n.t.
10	10a	31	1	0	32	0	0
	10b	30	2	0	32	0	0
11	11a	31	1	0	32	0	0
12	12a	29	3	0	32	0	0
15	15a	31	1	0	32	0	0

[a] N represents the number of subjects included in each group.
[b] One and 2 indicate the absence or the presence of the sequence variation, respectively, as compared to the LDLR sequence published by Yamamoto et al. (29).
[c] Not tested.

DGGE screening for sequence variations

In **Table 3** and **Table 4** all sequence variations detected in the FH population and in the control population are listed systematically and given a serial designation according to the exon where they occur.

Nucleotide alterations were revealed by the presence of two or more DNA bands on the DGGE gels compared with a single band in normal samples. As shown in **Fig. 1,** many alterations were characterized by the typical "four-band" pattern for heterozygotes, consisting of two upper bands representing the heteroduplexes and two lower bands representing the homoduplexes of mutant and normal DNA strands, respectively.

Polymorphisms

Seven variants fulfilled criteria that suggest that they represent polymorphisms, as they have been found both as homozygotes and heterozygotes in FH patients as well as in the control population (Table 3). To further test this hypothesis, the amplified fragments of the variant 8a, 12b, and 13a in the FH population were analyzed by conventional restriction fragment length polymorphism (RFLP) analysis with the enzymes *Stu*I, *Hinc*II, and *Ava*II, respectively. RFLP and DGGE analysis gave the same results, thus demonstrating that these variants represent known common polymorphisms (15, 18, 19). In case of the frequently occurring sequence variations 2b, 10c, 11b, and 15b, sequence analysis indeed confirmed that they represent common polymorphisms (14, 16, 17, 20).

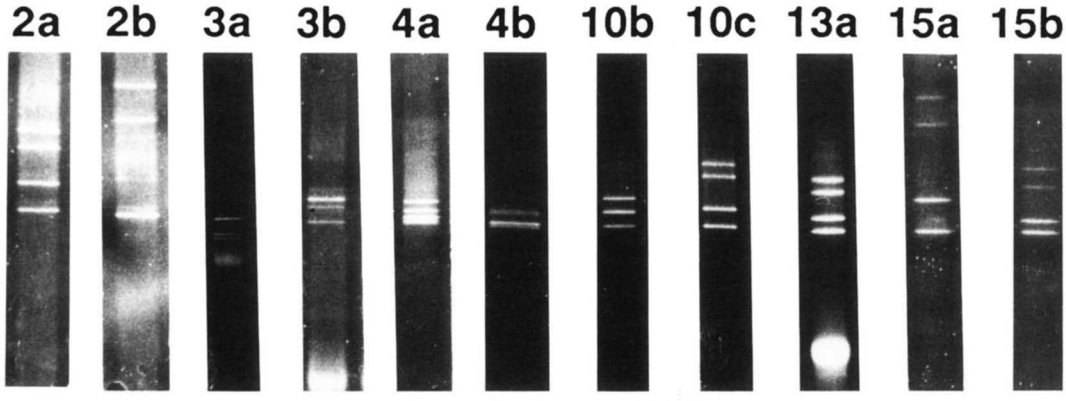

Fig. 1. Ethidium bromide staining of gels revealing some of the different sequence variations found. The designation of the sequence variations is indicated.

Point mutations

After DGGE analysis of all the 18 exons encompassing the LDL receptor protein coding and splice site consensus sequences in 32 heterozygous FH patients, we identified, in addition to the common polymorphisms, 28 aberrant DGGE patterns in 27 of the patients (Table 4). These variants appear only in the heterozygous form, suggesting that they represent candidate FH-causing mutations. In order to further test this hypothesis, 32 non-FH individuals were screened for the presence of these aberrant patterns in exons 2, 3, 4, 6, 10, 11, 12, and 15. None of these aberrant patterns was found in this control group (Table 4).

In total, 26 out of 27 patients had a sequence change, which could not be attributed to polymorphisms, in only one allele, while one patient (FH-868) had one change in each allele, in exon 3 (3b) and in exon 15 (15a). Thus the total number of aberrant DGGE patterns found is 28. Among these, five were located in exon 3 and 4, four in exon 6, three in exons 7, 10, and 12, two in exon 2, and one in exons 9, 11, and 15. In exons 1, 5, 14, 16, 17, and 18 we did not find any abnormal DGGE pattern, whereas in exon 8 and 13 only the common *Stu*I and *Ava*II polymorphisms were detected (Table 3).

Identical banding patterns found in some patients indicated that the same mutation might be present in apparently unrelated patients. This hypothesis was further confirmed by mixing the PCR products of patients who were expected to carry the same mutations, followed by heat-denaturation and reannealing at room temperature. The mixtures were tested again by DGGE. In patients expressing the same mutation no additional bands as compared with the original DGGE pattern were present. This reduces the total number of different sequence variations found to 16. Among these, 8 different DGGE aberrant patterns were found in either two or three genetically unrelated patients; 8 appeared to be unique (Table 4).

Direct solid-phase sequencing of the corresponding exon-specific PCR product revealed the nature of the molecular defects producing the aberrant DGGE patterns (**Table 5**). We identified six missense mutations (in exons 4, 6, 9, 10, 11, and 15), four splicing mutations (three in intron 3 and one in intron 9), three nonsense mutations (in exons 2, 7, and 4), two frameshift mutations (in exons 4 and 6) and one silent mutation (in exon 12). In summary, nine mutations occurred in the binding domain, six in the EGF precursor homology region and one in the O-linked sugars domain. Six corresponded to previously reported mutations (3); ten were novel mutations.

DISCUSSION

A considerable number of different mutations causing FH have been characterized. Part of these are large rearrangements, which can be easily detected by conventional Southern blot analysis (3). In the vast majority of FH patients, however, mutations could not be identified by this method, suggesting that they are mainly point mutations or small deletions or insertions. The wide heterogeneity of the FH-causing mutations and the large size of the gene have retarded the screening and identification of point mutations (3). Direct sequencing of PCR fragments of all exons of the LDL receptor gene is very laborious, and heterozygosity of mutations might cause equivocal interpretation. Therefore, a screening method that enables us first to restrict the area (exon) of the gene to be sequenced is required. An early diagnosis of FH would allow a timely monitoring and treatment of this disorder. Moreover, it offers the possibility of studying the possible role of mutations in the LDL receptor gene in mild hypercholesterolemia.

TABLE 5. List of mutations

Exon	DGGE Designation	Number of Carriers	Nucleotide Change	Effect on Coding Sequence	Name	Type	Reference
2	2a	2	G→A at 131	Trp→stop 23	W23X (FH-Cincinnati 5)	Nonsense	3
3	3a	1	G→C at 313 + 1	5' splice donor	313 + 1 (G→C)	Splicing	#[a]
	3b	3	G→A at 313 + 1	5' splice donor	313 + 1 (G→A)	Splicing	#
	3c	1	T→C at 313 + 2	5' splice donor	313 + 2 (T→C)	Splicing	#
4	4a	3	C→A at 501	Cys→stop 146	C146X	Nonsense	#
	4b	1	delTG at 646	stop 195	645delTG	Frameshift	#
	4c	1	C→T at 530	Ser→Leu 156	S156L (FH-Puerto Rico)	Missense	3
6	6a	2	delG at 877	stop 348	877delG	Frameshift	#
	6b	2	C→T at 917	Ser→Leu 285	S285L (FH-Amsterdam)	Missense	3
7	7a	3	C→T at 1048	Arg→stop 329	R329X	Nonsense	#
9	9a	1	G→A at 1285	Val→Met 408	V408M (FH-Afrikaner 2)	Missense	3
10	10a	1	G→A at 1567	Val→Met 502	V502M (FH-Kuwait)	Missense	3
	10b	2	G→A at 1359-1	3' splice acceptor	1359-1(G→A)	Splicing	#
11	11a	1	G→T at 1603	Asp→Tyr 514	D514Y	Missense	#
12	12a	3	C→T at 1725	Leu→Leu 554	L554L	Silent	#
15	15a	1	C→T at 2177	Thr→Ile 705	T705I (FH-Paris 9)	Missense	3

[a] # Denotes a novel mutation.

In the present study, sequence variations were detected in exon and splice site consensus sequences by DGGE. DGGE does not allow for the exact identification of the variation observed: it only helps to define the region that requires sequence analysis. Accordingly, an unequivocal discrimination between neutral polymorphisms and disease-causing mutations cannot always be made. However, for seven variations, we obtained strong evidence that they represent common polymorphisms as they occur in both the heterozygous and homozygous form in the FH population as well as in the control population (Table 3). All other variations observed did not occur in the homozygous form and were not found at all in the control population, suggesting that they represent candidates for FH-causing mutations. Indeed, subsequent sequence analysis confirmed this hypothesis.

With the exception of the silent mutation found in exon 12 (L554L) all the remaining 15 mutations found are likely to be FH-causing mutations. Five of them are missense mutations that have been reported elsewhere. In addition, we found a new missense mutation in exon 11 that changes an aspartic acid into a tyrosine residue. The other 10 mutations shown in Table 5 are splicing, nonsense, or frameshift mutations, which are all predicted to produce either aberrant splicings or prematurely truncated proteins and, ultimately, receptor proteins with a severely impaired function. The observation that most of the mutations found are of a type that severely disrupts the structure and function of the protein is not surprising, considering the fact that the patients included in our study displayed rather extreme LDL cholesterol levels (above 9.5 mmol/l). The fact that the mutations we have found are scattered along the entire LDLR gene confirms the wide heterogeneity of FH-causing mutations (3).

In exon 12, three patients producing the same DGGE pattern were found. Sequence analysis revealed that the aberrant pattern resulted from a C to T transition at nucleotide position 1725. This does not change the corresponding amino acid. Therefore this can only be classified as a silent mutation. However, this was the only sequence alteration found in the entire gene, except for the common polymorphisms, and it appears to occur in the FH population only. We therefore postulate that it is linked to some other rearrangement occurring in nonexon areas of the gene that have not been screened. Some of the mutations found occurred in more than one patient. This represents an additional advantage offered by DGGE: because each sequence alteration produces a distinctive, unique banding pattern, DGGE analysis enables us to identify recurrent mutations and to compare the pattern of newly identified mutations with that produced by known ones.

Eight different mutations have been found in at least two patients. This suggests that some specific mutations might be fairly frequent in the Dutch population (10). Although all subjects were apparently unrelated, a deeper genealogic investigation might reveal a common ancestry of some patients.

In exons 1, 5, 14, 16, 17, and 18 we did not find any abnormal DGGE pattern, whereas in exons 8 and 13 only the common *Stu*I and *Ava*II polymorphisms were detected. This indicates that, at least in these two exons, the DGGE assay itself did not represent a limit for the detection of sequence changes.

In 5 out of the 32 heterozygous FH patients included in this study we did not identify any aberrant pattern, except well-characterized polymorphisms. The reasons for this might be as follows. *i)* Lack of full resolution power of DGGE itself. It cannot be excluded that certain sequence changes might escape detection by this technique, although the melting maps of each fragment predicted that almost all, if not all, possible sequence variations should be identifiable. In this respect, a further demonstration of the resolving power of DGGE is offered by the observation that two different nucleotide alterations (3a and 3b) occurring at the same position (313 + 1) produced clearly distinct patterns, and a third alteration occurring at the adjacent nucleotide (313 + 2) also produced a different pattern. *ii)* The mutations might be in regions of the LDL receptor gene that have not been analyzed in the present study, such as the promoter region and most of the 3'-untranslated region of exon 18. However, previous studies have shown that mutations in the promoter region (from position −512 to −66) of the LDL receptor are either absent (30) or at least very rare (3). Mutations involving specific sequences in the 3'-untranslated region of the LDL receptor gene might also give origin to FH (31, 32). Moreover, novel important regulatory sequences, even in an intron further away from the LDL receptor gene cannot be ruled out. *iii)* In a minority of patients with a clinical diagnosis of FH also the involvement of genes other than the LDL receptor has to be considered. This could still be the case in the FH population we have selected, although patients carrying the gene for familial defective apoB (B3500) were excluded from this study.

In conclusion, in this study we have shown that DGGE is a reliable and rapid screening method for the detection of the presence of sequence variations in the LDL receptor gene. DGGE facilitates the characterization of the FH-causing mutations by restricting the region of the gene that requires subsequent sequence analysis, thereby facilitating the introduction of rapid DNA diagnosis for this disorder. Considering the high capacity and sensitivity, this method might also contribute to elucidate the possible role of LDL receptor mutations in mild hypercholesterolemia.

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