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Use of the denaturing gradient gel electrophoresis (DGGE) method for mutational screening of patients with Familial hypercholesterolaemia (FH) and Familial defective apolipoprotein B100 (FDB)

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Abstract

Familial hypercholesterolaemia (FH) and Familial defective apolipoprotein B100 (FDB) are autosomal dominant inherited diseases of lipid metabolism caused by mutations in the low density lipoprotein (LDL) receptor and apolipoprotein B100 genes. FH is clinically characterised by elevated concentrations of total cholesterol (TC) and low density lipoprotein cholesterol (LDL-C), presence of xanthomata and premature atherosclerosis. Both conditions are associated with coronary artery disease but may be clinically indistinguishable. Seventy-two (72) FH patients were diagnosed based on the Simon Broome's criteria. Mutational screening was performed by polymerase chain reaction (PCR)-denaturing gradient gel electrophoresis (DGGE). Positive mutations were subjected to DNA sequencing for confirmation of the mutation. We successfully amplified all exons in the LDL receptor and apo B100 genes. DGGE was performed in all exons of the LDL receptor (except for exons 4-3', 18 and promoter region) and apo B100 genes. We have identified four different mutations in the LDL receptor gene but no mutation was detected in the apo B100 gene. The apo B100 gene mutation was not detected on DGGE screening as sequencing was not performed for negative cases on DGGE technique. To our knowledge, the C234S mutation (exon 5) is a novel mutation worldwide. The D69N mutation (exon 3) has been reported locally while the R385W (exon 9) and R716G (exon 15) mutations have not been reported locally. However, only 4 mutations have been identified among 14/72 patients (19.4%) in 39 FH families. Specificity (1-false positive) of this technique was 44.7% based on the fact that 42/76 (55.3%) samples with band shifts showed normal DNA sequencing results. A more sensitive method needs to be addressed in future studies in order to fully characterise the LDLR and apo B100 genes such as denaturing high performance liquid chromatography. In conclusion, we have developed the DNA analysis for FH patients using PCR-DGGE technique. DNA analysis plays an important role to characterise the type of mutations and forms an adjunct to clinical diagnosis.

Key words: Familial hypercholesterolaemia, LDL receptor gene, apo B100 gene, denaturing gradient gel electrophoresis

INTRODUCTION

Familial hypercholesterolaemia (FH) is a common inherited disorder of lipoprotein metabolism associated with premature vascular disease.[1] It is caused by different mutations of the low density lipoprotein (LDL) receptor gene.[2] To date, more than 700 mutations have been identified worldwide.[3] The prevalence of heterozygotes among European, American and Japanese populations is about 1 in 500 individuals and for homozygotes 1 in 1 million individuals in the United States.[4] However, in some culturally and geographically isolated population groups, the frequency of the disease and of specific mutations is much higher as a result of the founder effect such as that observed in French Canadians, Sephardic Jews, Lebanese Christian Arabs, South African Afrikaners and Finns.[5,6,7,8] LDL receptor gene mutations in our Malaysian population have not been fully characterized. FH is characterised by severe elevation of the

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LDL-C level, the major cholesterol transported in human plasma. This in turn leads to deposition of LDL-derived cholesterol in tendon (xanthomata) and in arteries (atheromas).[9] It is inherited by an autosomal dominant trait with a gene dosage effect where homozygotes are more severely affected than the heterozygotes.[9] Conventionally, FH is diagnosed based on clinical and biochemical criteria such as elevated plasma LDL cholesterol levels, presence of xanthomata and a strong family history of premature coronary artery disease (CAD) as stated by the Simon Broome's criteria.[9] However, physical signs and biochemical features of FH may not be present in younger patients hence delaying the clinical diagnosis. Therefore, molecular diagnosis may have a role to ascertain the diagnosis among family members. Early detection of FH will allow preventive measures be initiated at younger age to reduce morbidity and mortality due to CAD and sudden cardiac death (SCD).

Familial defective apolipoprotein B100 (FDB) is an autosomal dominant disorder of lipoprotein metabolism also caused by impaired binding of LDL particles to LDL receptors on the surface of liver cells. Diminished uptake of LDL by the liver results in a twofold to threefold elevation of plasma LDL cholesterol level. Patients with FDB, therefore, are likely to bear a substantially higher risk of premature atherosclerosis and its subsequent cardiovascular complications. The deficient binding of LDL to its receptor is caused by an amino acid substitution in the putative LDL-receptor binding domain of the mature apolipoprotien B-100 (apo B100) protein. A substitution of adenine for guanine at nucleotide position 10699 of the apo B gene results in the substitution of glutamine for arginine at amino acid position 3500. This mutation is referred to as the $apoB_{3500}$ mutation and is clinically milder compared to FH. The objective of this study was to screen for LDLR gene mutations using the denaturing gradient gel electrophoresis (DGGE) followed by direct sequencing in patients clinically diagnosed as FH.

MATERIALS AND METHODS

Study population

A total of 72 FH patients from 39 unrelated families, [mean ± SD age = 41.3 ± 1.8, range 5-68 years] attending the specialist clinics at the Hospital Universiti Kebangsaan Malaysia and the National Heart Institute, Kuala Lumpur were studied. This cohort consisted of patients from the three main local ethnic groups: 58 Malays (28 families), 13 Chinese (10 families) and 1 Indian (1 family). The FH patients were classified into definite and possible FH based on the Simon Broome's criteria.[9] Definite FH was defined as serum total cholesterol (TC) levels of $\geq$ 7.5 mmol/L and/ or LDL cholesterol of $\geq$ 4.9 mmol/L (for age > 16 years), TC > 6.5 mmol/L (for age $\leq$ 16 years) and tendon xanthomata either in the patients him/herself or at least 1 other family member (first or second degree relatives). Possible FH was defined as similar biochemical criteria (as above) and family history of premature CAD and/or hypercholesterolaemia, (TC $\geq$ 7.5 mmol/L) in first degree (age < 60 years) or second degree relatives (age < 50 years). Whole blood samples were collected in EDTA-anticoagulated tubes and fasting sera were collected in plain tubes after an overnight fast. Blood samples from normocholesterolaemic controls (TC< 5.2 mmol/L) were also collected in parallel. Samples of known LDL receptor and apo B-100 gene mutations were also obtained from the other FH research centre at the National Heart Institute . This study complied with the declaration of Helsinki[10] and the protocol was approved by the Research Ethics Committee of the Faculty of Medicine UKM. All patients gave written informed consent for participation in this study.

Lipid determination

All patients undertook a screening protocol consisting of clinical history, physical examination and laboratory tests including fasting glucose (FG), fasting serum lipids (FSL), renal profile (RP), liver function tests (LFT) and thyroid function tests (TFT). Blood samples were analysed for the above tests using standard techniques. Fasting plasma samples were analysed for glucose using the hexokinase enzymatic reference method on an automated analyser (Cobas Integra 700, Roche Diagnostics, Basal, Switzerland). TC, TG and HDL-C were measured by enzymatic reference methods on an automated analyzer (Cobas Integra 700, Roche Diagnostics, Basal, Switzerland). LDL-C concentration was derived by calculation using the Friedwald equation, LD-C = TC - (HDL-C + TG/2.18).[11]

DNA extraction

Total genomic DNA was prepared using a commercial kit (DNA isolation kit for mammalian blood) (Roche Diagnostics, Basal, Switzerland) using a method as described by the manufacturer.

The DNA samples were electrophoresed on 1.2% agarose and purity was determined using a spectrophotometer (A260/A280).

Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE)

The 3456-3553 region of the apo B100 and all 18 exons of the LDL receptor including the promoter region were screened in all patients. Exon 4 was divided into 2 parts including the 4-3' and 4-5' regions. In total, 21 sets of primers were used in this study. PCR was performed using oligonucleotide primers as previously described by Nissen *et. al.* (1996)[12] as shown in Table 1. For the DGGE, the GC clamps were incorporated either in the forward or reverse primer to aid the formation of uniform low melting domains. We attached 40 to 50 bp GC clamps as described by Nissen *et. al.* (1996). PCR mixtures were prepared in a total volume of 50 µl consisting of: 100-500ng DNA, 1.5-3.0 mM $MgCl_2$, 0.2mM dNTPs, 10x PCR Buffer, 10-20 pmol oligonucleotide primers and 2.5U taq polymerase (Platinum Taq, Invitrogen). PCR was carried out using the iCycler Thermal Cycler (Bio-Rad, USA). DNA template was initially denatured for 5 minutes at 94°C, followed by 35 cycles as follows: denaturation for 15 to 30 seconds at 95°C, annealing for 13 to 45 seconds at 55°C to 70°C and elongation for 13 to 60 seconds at 72°C and followed by final extension at 72°C for 5 to 10 minutes. These conditions were optimised individually for each amplicon of the gene. DGGE was performed by the DCODE Universal Mutation Detection System from BioRad Laboratories, USA to identify single base changes in a segment of DNA. PCR products were heated at 95°C for 5 minutes, then placed at 65°C for 1 hour and slowly cooled at room temperature to generate the heteroduplex molecules. The PCR products of particular samples with their heteroduplex molecules were electrophoresed together with 6% gel and 20-80% denaturing solutions (depending on the exons), at 130V, 60°C for 4 to 6 hours (depending on exons). Finally, the gel was stained with ethidium bromide and visualized with a digital camera system (Kodak)(Figures 1, 2 and 3).

Confirmation of mutation by DNA sequencing

Samples with band shifts were subjected to DNA sequencing for confirmation of the mutations. PCR was repeated using oligonucleotide primers without GC clamps. PCR products were purified using QIAquick gel extraction kit (Qiagen, Germany) and subsequently were electrophoresed on 1.2% agarose gel at 100V for 1 hour. The cycle sequencing was performed at 96°C for 10 minutes, 50°C for 5 seconds and 60°C for 10 seconds for 25 cycles. Finally, the cycles sequencing products were purified using ethanol/sodium acetate precipitation method. The DNA was suspended in the HI-DI formamide and DNA sequencing was performed using the ABIPRISM 3100 Genetic Analyzer. The results were analyzed using BLAST (http://www.ncbi.nlm.nih.gov/).

RESULTS

All the exons of the LDL receptor, promoter and the apo B100 genes were successfully amplified. We also successfully performed the DGGE on the apo B100 gene and 17 exons of the LDL receptor gene. For exons 4-3', 18 and the promoter region of the LDLR gene, direct sequencing was done to fully characterize the mutations in these regions due to difficulties in optimising the technique. In total, 76 band-shifts were identified in several exons by the DGGE. We have identified 4 mutations involving the LDL receptor gene while no mutation of the apo B100 gene was detected. These were the D69N (exon 3), C234S (exon 5), R385W (exon 9) and R716G (exon 15). D69N mutation in exon 3 has been found in 1 FH patient, C234S in 9 definite FH patients, R385W mutation in 2 patients with possible FH and R716G in 1 patient with definite FH. These four types of mutations were identified in 4 different families. Several non-mutational single nucleotide polymorphisms (SNPs) were also identified in exons 10, 11, 12 and 13 which involved substituition of the nucleotide sequence with no changes in the amino acid sequence. The DNA sequencing results are as shown in figures 4, 5 and 6. Table 2 and 3 illustrates the mutations and single nucleotide polymorphisms (SNPs) that have been identified using the DGGE technique.

In total, 76 band-shifts were detected by the DGGE in exons 3, 4-5', 5, 9, 10, 11, 12, 13 and 15. However, sequencing analysis for confirmation of the mutations revealed 4 different mutations and 4 single nucleotide polymorphism (SNP) in the LDLR gene while the remaining was normal. This implied that the DGGE method is sensitive but not highly specific.

Sensitivity of the DGGE usually results in a mutation detection rate of over 80%.[14] We could not assess this in our study as we only sequenced those with band shifts present on the

TABLE 1: Oligonucleotide primers for amplification of the promoter region and the 18 exons of the human LDL receptor gene and the codon 3500 region of the human apolipoprotein B100 gene

LDLR	Forward Primer (5')	Reverse Primer (3')	Base pair	DGGE
1.Promoter	[b]AGGACTGGAGTGGGAATCAGAGC	TGCTGTGTCCTAGCTGGAAACCC	252	-
2.Exon 1	[a] TTGAAATGCTGTAAATGACGTGG	CTGGCGCCTGGAGCAAGC	256	30-70
3.Exon 2	[b] CGTGGTCAGTTTCTGATTCTGGCG	ATAAATGCATATCATGCCCAAAGG	253	30-70
4.Exon 3	[b] TCGGCCTCAGTGGGTCTTTC	ACTCCCCAGGACTCAGATAGGC	268	30-70
5.Exon 4-5'	[b] ACTGCGGCAGCGTCCCCGGC	GGATGCAGGTGGAGCTGTTGC	297	20-80
6.Exon 4-3'	ACCTGTGGTCCCGCCAGC	[b] CCAGGGACAGGTGATAGGACG	345	-
7.Exon 5	[b] GGCCCTGCTTGTTTTTCTCTGG	AGCAGCAAGGCACAGAGAATGG	282	20-80
8.Exon 6	[b]ACGAAACTGAGGCTCAGACACACC	GCTCCCCACAAACTCTGCAAGC	262	20-80
9.Exon 7	[b] AGAGTGACCAGTCTGCATCCCTGG	TTGGTTGCCATGTCAGGAAGC	253	35-65
10.Exon 8	[b] TCCCCACCAAGCCTCTTTCTCTC	CCACCCGCCGCCTTCC	222	30-70
11.Exon 9	[C] CTGACCTCGCTCCCCGGACC	GGCTGCAGGCAGGGGCGACG	278	30-70
12.Exon 10	GCAGTGAGATGAGGGCTCCTGG	[b] CCTGCAGCCCTCAGCGTCG	349	30-70
13.Exon 11	[b] GGATCCTCCCCCGCCCTC	TGGCTGGGACGGCTGTCC	239	30-70
14.Exon 12	GGCCCTCAGGCCCTCTGG	[b] CCGAGTTTTCTGCGTTCATCTT	336	30-70
15.Exon 13	[a] GTCATCTTCCTTGCTGCCTG	CACAAGGAGGTTTCAAGGTTGG	264	20-60
16.Exon 14	[a] TCTCGTTCCTGCCCTGACTCC	GACACAGGACGCAGAAACAAGG	274	30-70
17.Exon 15	[b] GGCACGTGGCACTCAGAAGACG	[a] GTGTGGTGGCGGGCCCAGTC	288	30-70
18.Exon 16	[a] CTCCATTTCTTGGTGGCCTCCC	CATAGCGGGAGGCTGTGACCTGG	239	30-70
19.Exon 17	[a] GGGCAGCTGTGTGACAGAGCG	CATGGCTCTGGCTTTCTAGAGAGG	279	30-70
20.Exon 18	[a]CCTGAGTGCTGGACTGATAGTTTCC	AAGGCCGGCGAGGTCTCAGG	190	-
21.Apo B100	[b] GGAGCAGTTGACCACAAGCTTAGC	GGTGGCTTTGCTTGTATGTTCTCC	382	30-70

[a] 50-bp GC-clamp: CGCCCGCCGCCGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG
[b]40-bp GC-clamp: CGCCCGCCGCGCCCCGCGCCCGTCCCGGCCGCCCCCCCGCCCG
[c]46bpGC/10bpAT:CGCCCGCGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCGAAATAATAA
[d]3-bp GC-clamp: CGG
[e] Apolipoprotein B codon 3456-3553 (Nissen *et al*., 1995c)

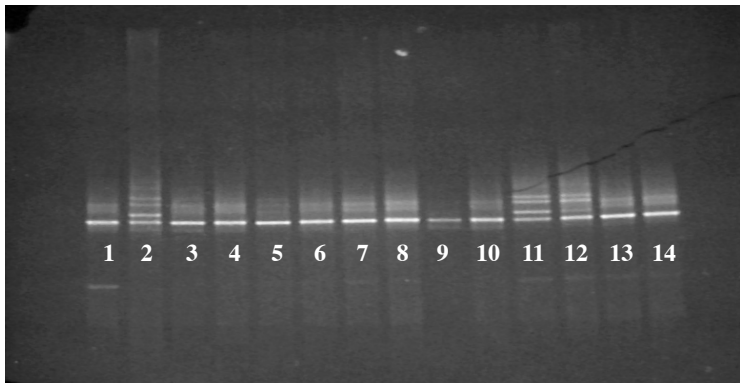

FIG. 1 : DGGE analysis of exon 3 of the LDL receptor gene. Lane 1 represents the normal control, lane 2 represents the positive control of R60C mutation, lanes 3 - 14 are samples of FH patients. Lanes 11 and 12 shows positive band shifts suggesting the presence of mutations

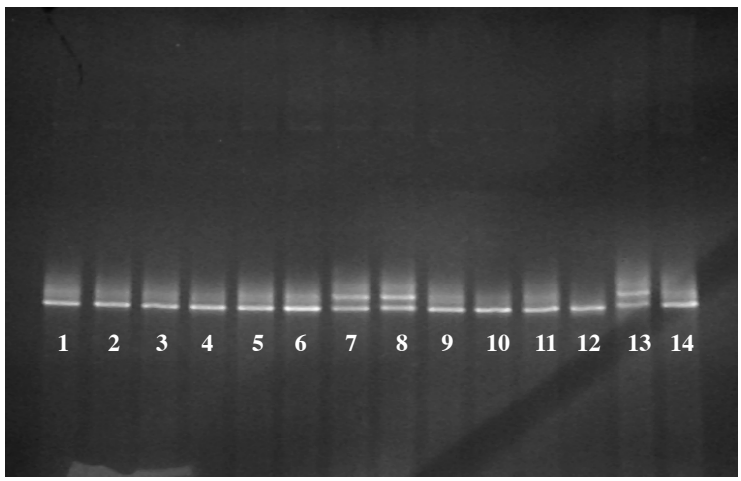

FIG. 2 : DGGE analysis of exon 5 of the LDL receptor gene. Lane 14 is the normal control, lane 13 the positive control with D245E mutation whilst lanes 1 - 12 are samples of FH patients. Lanes 7 and 8 show band shifts suggesting presence of mutations in this patient.

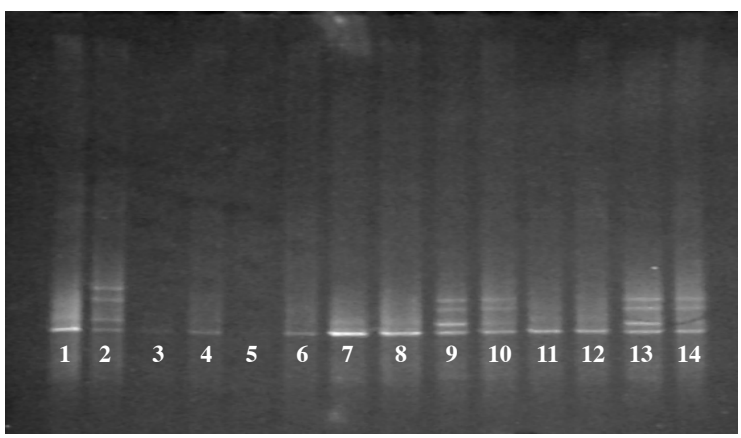

FIG. 3 : DGGE analysis of exon 9 of the LDL receptor gene. Lane 1 is a normal control, lane 2 a positive control (V408M mutation) whilst lanes 3 - 14 are samples of FH patients. Lanes 9, 10,13 and 12 showed band shifts suggesting presence of mutations in these patients.

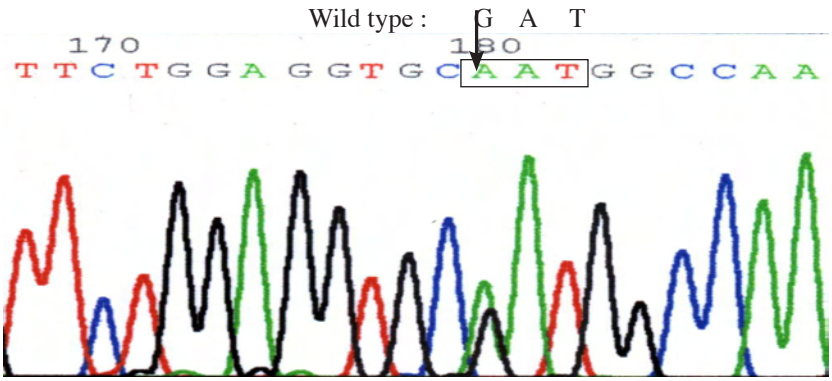

FIG. 4 : DNA sequencing results of exon 3 of the LDLR gene showing the base change from G → A at nucleotide 268 resulting in substitution of amino acid aspartic acid (D) to proline (N) at codon 69, designated as D69N mutation. This is an example of a heterozygous mutation (as demonstrated by two peaks).

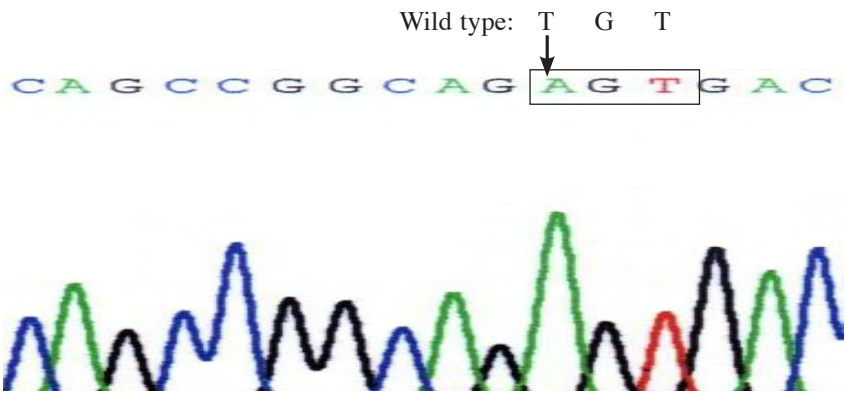

FIG. 5 : DNA sequencing results of exon 5 of the LDLR gene showing the base change from T → A at nucleotide 763 resulting in substitution of amino acid cysteine (C) to serine (S) at codon 234, designated as C234S mutation. This is an example of a homozygous mutation.

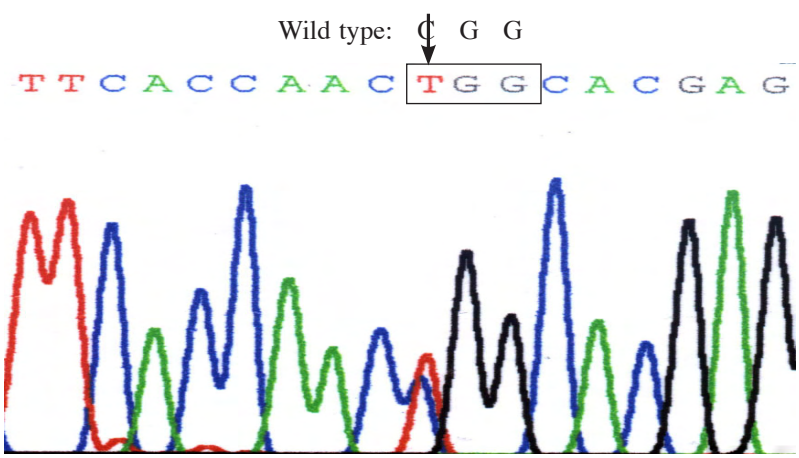

FIG. 6 : DNA sequencing results of exon 9 of the LDLR gene showing the base change from C → T at nucleotide 1216 resulting in substitution of amino acid arginine (R) to tryptophan (W) at codon 385, designated as R385W mutation.

TABLE 2 : Mutations that have been identified using DGGE technique

Mutation	Patient	Diagnosis	Exon	Status*	Event	Effect
D69N	FH 074	Possible FH	3	Htz	G → A at nucleotide 268	Asp → Asn at codon 69
C234S	FH 001 FH 002 FH 010 FH 013 FH 008 FSK 001 FSK 002 FSK 003 FSK 004	Definite FH Definite FH Definite FH Definite FH Definite FH Definite FH Definite FH Definite FH Definite FH	5	Hz Htz Htz Htz Htz Hz Htz Htz Htz	C → S at nucleotide 234	Cys → Ser at codon 234
R385W	FSN 001 FH 032	Possible FH Possible FH	9	Htz Htz	C → T at nucleotide 1216	Arg → Trp at codon 385
R716G	FH 063	Definite FH	15	Hz	A → G at nucleotide 2209	Arg → Gly at codon 716

* Htz is heterozygous mutation, Hz is homozygous mutation

TABLE 3 : Single nucleotide polymorphisms (SNPs) that have been identified using DGGE technique

SNPs	Patient	Exon	Event	Effect
R450R	FH 081 FH 096 FSJY 001 FH 023	10	G → A at nucleotide 1413	Arg → Arg at codon 450
N510N	FSY 002 FH 018 FSH 004 FSY 001	11	C → T at nucleotide 1617	Asn → Asn at codon 510
N570N	FSZ 001 FH 026 FH 037 FH 013 FSR 001	12	T → C at nucleotide 1773	Asn→ Asn at codon 570
V632V	FH 041 FH 081 FH 003 FH 013 FSH 005 FSC 001 FH 045	13	C → T at nucleotide 1959	Val → Val at codon 632

DGGE. However, 4 mutations were identified among 14/72 patients (19.4%) and 39 FH families (10.3%). Specificity of this technique was 44.7% based on the fact that 42/76 (55.3%) samples with band shifts showed normal DNA sequencing results.

DISCUSSION

We have successfully established and used the DGGE technique to screen for mutation in the LDLR gene amongst a group of FH patients. As described earlier the diagnosis of FH by conventional ways may be associated with some uncertainties since 15-20% of the relatives could be missed by cholesterol testing alone. Hence, genetic diagnosis of FH and their relatives may play a role in providing a definite diagnosis of FH and counseling of the family members.

At the time of performing the study, the two most widely used techniques from mutational screening are the single-strand conformation polymorphism (SSCP) and the denaturing gradient gel electrophoresis (DGGE).[3,13] We chose the DGGE approach as it is considered to be equally sensitive compared to SSCP but less laborious in nature.[14] In DGGE, double stranded DNA is subjected to an increasing denaturant environment and will melt in discrete segments called "melting domain." The melting temperature (Tm) of these domain is sequence-specific. When the T_m of the lowest melting domain is reached, the DNA will become partially melted, creating branched molecules. Partial melting of DNA reduces its mobility in a polyacrylamide gel. Since the T_m of a particular melting domain is sequence-specific, presence of mutation will alter the melting profile of that DNA compared to wild-type. DNA containing mutations will encounter mobility shifts at different positions in the gel compared to the wild-type.

In total, 76 band-shifts were detected by the DGGE in exons 3, 4-5', 5, 9, 10, 11, 12, 13 and 15. However, sequencing analysis for confirmation of the mutations revealed 4 different mutations and 4 single nucleotide polymorphism (SNP) in the LDLR gene while the remaining was normal. This implied that the DGGE method is sensitive but not highly specific.

There are several advantages and disadvantages in using DGGE as a tool for mutational screening. The most crucial factor in the DGGE was in the optimization of the conditions. The percentage of denaturing solution had to be determined first by the perpendicular DGGE to determine the optimal condition for the subsequent parallel DGGE. We eventually performed a parallel DGGE according to Nissen et al. (1999) with slight modification of the percentage of the gel and denaturing solution concentration. Having had experience in using the SSCP approach for other studies, we found that the DGGE analysis for mutational screening was also a laborious method to perform. In addition it requires a special gel electrophoresis system. Finally, this method also has a low throughput, i.e. 32 samples per electrophoretic run. Introduction of the GC clamp (40-50bp) was necessary to increase method sensitivity, but this contributed to the increased in the total cost. Additional primers without the GC clamp may be required for the sequencing analysis.

The sensitivity of the DGGE usually results in a mutation detection rate of over 80%.[14] We could not access this in our study as we only sequenced those with band shifts present on the DGGE. However, 4 mutations were identified among 14/72 patients (19.4%) and 39 FH families (10.3%). Specificity (1-false positive) of this technique was 44.7% based on the fact that 42/76 (55.3%) samples with band shifts showed normal DNA sequencing results. This may be due to the background of the PCR products which was carried over to the DGGE analysis. As a result, more than 1 band was produced and detected as possible mutation in the sample.

In conclusion, we have successfully established a screening method for FH patients using the PCR-DGGE approach. Four different LDL receptor mutations (2 of which were novel mutations) were identified in 19.4% of patients and 10.3% of unrelated families. Four different non-mutational SNP's were identified in 4 other exons. Future studies should address mutational screening in FH patients using a more rapid and sensitive method and characterisation for other potential genes causing a clinical disorder similar to FH.

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