Utilities for high throughput use of the single strand conformational polymorphism method: screening of 791 patients with familial hypercholesterolaemia for mutations in exon 3 of the low density lipoprotein receptor gene

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Abstract

We have modified several aspects of the single strand conformational polymorphism (SSCP) method to increase the speed with which the technique can be used for mutation detection. The methods attain high resolution of small mobility differences using long (30 cm) gels and use a modified polymerase reaction to maximise detection sensitivity using a minimised quantity of ^{32}P . By using custom cut "sharktooth" combs (4.5 mm between teeth) as the slot formers, commercially available multichannel pipettes (9 mm tip to tip) can be used to load eight or 12 samples at a time from standard microtitre plates. PCR products that have been prepared and radiolabelled using simplified protocols are loaded on to the gel, and after a precalculated time of electrophoresis another set of samples can be loaded, either with combs moved across 2.25 mm or onto the same gel tracks. The run conditions are calculated so that there is no overlap between the bands produced by the two loadings, thus doubling the amount of information that can be gained from one gel. A computer program has been developed to solve equations to determine suitable timings for repetitive loadings. Finally, a modification of the gel pouring system is described so that two gels can be poured between three standard glass plates, with both gels run simultaneously. Of the order of 1000 PCR reactions can be prepared and analysed in 24 man hours using five 40 cm $\times$ 30 cm gel tanks. The application of these techniques is described to detect SSCPs in exon 3 of the low density lipoprotein receptor (LDLR) gene in 791 patients with familial hypercholesterolaemia (FH). Eight different SSCP patterns were seen, one of which was caused by the previously described E80K mutation, which was present in 11 patients (1.4%). In total, 32 patients (4%) were identified with exon 3 mutations.

dominant pattern of inheritance.¹ It is characterised clinically by a raised concentration of low density lipoprotein (LDL) cholesterol in blood, tendon xanthomata, and an increased risk of myocardial infarction. Based on the estimated population frequency of carriers of 1/500, there are more than 110 000 FH heterozygotes in the UK, of whom an estimated maximum of 80% and probably fewer than 30 to 50% are even known to have hypercholesterolaemia: fewer than 0.1% have been confirmed at the genetic level to carry a gene for familial hypercholesterolaemia. The hyperlipidaemia and arterial lesions of these patients are responsive to treatment by diet and drugs,^{2,3} and such treatment is likely to reduce subsequent morbidity and mortality. Although management is based on the phenotype, counselling and future genetic tracing programmes will be helped by knowledge of the genotype so that definitive genetic tests can be offered. Genetic characterisation would thus be helpful both for research and in the longer term as a diagnostic tool.

FH commonly results from a genetic defect in the low density lipoprotein (LDL) receptor which controls the uptake of plasma LDL,¹ and worldwide more than 140 different mutations of the LDLR gene have been characterised at the DNA level.⁴ Within a geographically or culturally isolated population, or where a large proportion of people are related by descent because of migration, there may be a single mutation causing FH in many of the patients.⁵⁻⁷ In the UK, where there is a very heterogeneous population, it is unlikely that any mutations will be present at a high frequency in FH patients. Our findings to date in a sample of 200 FH patients from London are that 5% of patients have a gross rearrangement,^{8,9} while in a further 18% a small deletion or a single base mutation has been detected in exon 3, 4, or 14.¹⁰⁻¹² Thus either many further mutations must be found, or a different strategy must be developed, if DNA methods are ever to become a useful adjunct to classical screening methods.

Several methods have been published which allow rapid comparison of the sequence of specific fragments of DNA amplified in vitro by the polymerase chain reaction (PCR) from different subjects. These include¹³ the chemical cleavage of mismatched bases and the use of

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Familial hypercholesterolaemia (FH) is a common inherited disease showing an autosomal

denaturing gradient gel electrophoresis, both of which are slow and technically demanding, but which appear to detect all possible mutations. A third method is the single strand conformational polymorphism (SSCP) technique, which is based on the fact that single base changes result in conformational changes in single stranded DNA allowed to fold under non-denaturing conditions, which can be detected as a different pattern of migration on a polyacrylamide gel.¹⁴ DNA fragments are usually labelled by inclusion of ³²P dCTP in the amplification mixture and subsequent detection by autoradiography. This method has been more widely used than the other two, in part because it is rapid and does not use toxic chemicals or require a hybridisation step. The ability to detect differences in the migration pattern is greatest in fragments under 300–400 bp. Some studies have reported that the method does not detect all single base changes,¹⁵ and sensitivity to polymorphism can sometimes be improved for particular PCR products by a change of conditions. Our experience and that reported by others suggests that the method detects 80% to 90% of all single base changes,^{22,16} but this is probably dependent on the sequence under study and the analytical conditions used. This method has been used successfully to detect six different mutations in the 3' half of exon 4 of the LDL-R gene.¹⁷ All of these give a pattern of fragments distinguishable from that seen with the normal DNA. We report here a number of modifications of standard SSCP protocols, which will improve the throughput of the technique by an order of magnitude, enabling a more comprehensive approach to the study of the molecular genetics of FH in hundreds of patients, or of any other disorder amenable to study by SSCP.

Materials and methods

PATIENTS

A total of 791 patient samples were included in this study. Many patients or samples have been the subject of earlier clinical studies or studies of exon 4,^{9,11,17} including 200 from London lipid clinics, and a variety from other centres in the UK. A further 150 from Southampton and South West Hampshire District (L B Day, I N M Day, unpublished data), and a wide range of samples sent in by other clinicians were included in this development of a practicable high throughput system for molecular genetic study of FH. All patients fulfilled clinical diagnostic criteria for "definite", "probable", or "possible" FH, and with approximately 20 exceptions are believed not to be first or second degree relatives of each other.

ISOLATION OF DNA

Genomic DNA was isolated from frozen whole blood, using serial steps of cellular lysis with a sucrose buffer, nuclear lysis including sodium dodecyl sulphate and overnight protein digestion by proteinase K, salt precipitation of residual debris, and ethanol precipitation of DNA, as previously described.¹⁸

Improved protocols for high throughput PCR and SSCP

PCR

Exon 3 was amplified by PCR using primers flanking exon 3. The primers were 25 bases in length. The sequence of the 5' primer is 5'-TGACACTTCAATCCTGTCTCTTCTG and for the 3' oligonucleotide 5'-ATAGCAAAGGCAGGGCCACACTTAC, to give a product of 172 bp.⁴ Oligonucleotides were obtained from Genosys, USA. The amplifications were performed in an automated thermal cycler (Hybaid Omnigene) using *Thermus Aquaticus* (*Taq*) DNA polymerase (Gibco BRL, Paisley, UK) in a total volume of 20 µl and overlaid with 20 µl paraffin oil, using the PCR cycling conditions 96°/five minutes, 68°/five minutes for one cycle, 68°/five minutes for 35 cycles, and 68°/five minutes for one cycle.⁴

PCR SETUP

Microtitre arrays of dried template DNA

DNA samples were diluted to a concentration of approximately 10 ng/µl in 0.5 ml in 96 well arrays, the master plate. Of this, 2.5 µl was then transferred to 96 well microtitre plates (Hybaid Omnigene microtitre plates for PCR) using an eight channel multipipette. Many copies of this plate can be made, left to dry overnight, and stored at room temperature for future use.

Minimisation of ³²P usage

For reasons discussed below, we elected to use ³²P for detection of bands on SSCP gels. To minimise use of radioisotope, a modified PCR polymerase mix containing one tenth the usual concentration of dCTP was used (0.2 mmol/l dATP etc, 0.02 mmol/l dCTP), in conjunction with a ten-fold reduction (from 0.3 to 0.03 µCi/PCR) in necessary concentration of [α -³²P]dCTP compared with earlier protocols.^{14,19}

The complete polymerase mix including oligonucleotides (0.8 pmol/PCR), *Taq* polymerase (0.2 U/PCR), deoxynucleotides (0.2 mmol/l dATP etc, 0.02 mmol/l dCTP), buffer (10 mmol/l Tris pH 8.5, 50 mmol/l KCl, 0.0015 w/v gelatin), w1 (0.05% w/v), and radioisotope (0.03 µCi[α -³²P]dCTP/PCR) was premixed for distribution into wells of plates containing dried DNA templates.

SSCP

PCR product (2 µl) was added to 12 µl of a 7:5 ratio mix of formamide dye mix (95% formamide, 10 mmol/l EDTA, 0.025% bromophenol blue, 0.025% xylene cyanol FF) and a solution of 0.1% (w/v) sodium dodecyl sulphate (SDS) containing 10 mmol/l EDTA. The PCR DNA was denatured at 95°C for five minutes and chilled immediately on wet ice; 7.5 µl samples were loaded on to a 7.5% polyacrylamide non-denaturing gel (ratio of acrylamide to bisacrylamide of 49:1) in 1 × TBE buffer with 5% glycerol, 10 mmol/l EDTA. Gels were 40 cm × 30 cm × 0.4 mm. Electro-

phoresis was conducted at a constant voltage of 200 V overnight at room temperature (usually 21–25°C) until the xylene cyanole had migrated 20 cm. The gels were then transferred to Whatman 3 mm chromatographic paper, dried, and exposed to Hyperfilm MP (Amersham, UK) for one to four days at –70°C with one intensifying screen before film development.

SSCP SETUP

Double gels

Two gels are poured between one large glass sequencing plate (33 × 42 cm) and two smaller plates (33 × 39 cm), so that two gels can be subjected to electrophoresis simultaneously on one apparatus. Three plates are defined: “lowest”, which is the large plate and the outermost one when the arrangement is clamped to the vertical electrophoresis apparatus, “middle”, and “uppermost”. The terms refer to the plates as arranged horizontally on a levelling table for gel pouring. Before gel pouring, the four glass faces which will contact gel are silanised with Repelcote (BDH product number 63216 4J, BDH, Poole, UK), 2 ml spread evenly with the edge of another glass plate. The plates are arranged with their sides aligned, with a pair of 0.4 mm × 10 mm × 400 mm spacers supporting the sides of both the middle and uppermost glass plates. The edge of the middle plate which will contact the bottom gel tank is arranged to be recessed 5 mm relative to the other plates. This leaves a 5 mm “platform” at the top, which better facilitates the insertion of the comb for the upper gel. The three plates are clamped at the sides with bulldog clips. The bottom gel (on the large plate) is poured first (by injection between the plates using a large syringe, with firm tapping to avoid the formation of bubbles during pouring), the comb inserted, and the gel left to set for 30 to 60 minutes. The second gel is then prepared identically. The gels can be stored for several days with TBE saturated tissues and clingfilm covering the ends. Care must be taken when clamping the gels to the apparatus to ensure that no air bubbles are trapped in the recess between the outermost and innermost plates in the bottom tank.

Double loading gels

After first loading the gel, the samples were electrophoresed at 400 V until the bromophenol blue had run 2.5 to 3 cm, at which point the electrophoresis was stopped and a second set of SSCP samples was loaded in the same way as the first samples. Before loading the second time, the sharktooth comb was removed and repositioned half a well across from its original position. This enables the first and second loading bands to be distinguished more easily.

The first and second loading single and double strands need to be non-overlapping if the gel is to be informative. The calculation of the timing of the second loading is possible if the relative mobilities of the two single strands, one double strand, and marker dyes are already known for the set of conditions to be used.

These mobilities were determined in a previous experiment with single loading. In order that repetitive loading can be applied both to the same and different exons, we have prepared a computer simulation which enables the solution of equations relating relative mobilities, for boundary conditions including timing of loading, range of migration distance desired, and time delays between loadings (available from G P Weavind, 73064,3063@compuserve.com by email request). Double loading the gel means that a whole 96 well microtitre array of samples may be analysed on one gel.

Sharktooth comb

Sharktooth combs were custom cut with 4.5 mm spacing tooth to tooth to enable the use of a multichannel pipette compatible with microtitre plates (9 mm well to well spacing) for loading the SSCP gels. This results in every second well being loaded with a multichannel pipette so that two adjacent columns or rows of a microtitre plate are interleaved when loading.

SCREENING BY ALLELE SPECIFIC OLIGONUCLEOTIDES FOR THE E80K MUTATION IN EXON 3

PCR of exon 3 was carried out as for SSCP of exon 3 but without dCTP modification and radioisotopic incorporation as described above. The PCR products were checked by electrophoresis on 7.5% polyacrylamide. Aliquots (5 µl) of the PCR product were mixed with an equal volume (5 µl) of a denaturing solution (2 mol/l NaCl/1 mol/l NaOH) in 96 well microtitre array format and incubated at room temperature for 15 minutes. Duplicate nylon membranes (Hybond N+, Amersham International) were spotted with 4 µl aliquots of denatured product using an eight channel pipette to give duplicate filters in grids representing the original 96 well array. Then 30 pmol of the allele specific oligonucleotides (ASOs), (FH45 (Glu) 5' TCAGACGAGCAA and FH46 (Lys) 5' TCAGACAAGCAA) were end labelled and purified using Sephadex G-25 spun columns. The filters were prehybridised and hybridised in 5 × SSC, 5 × Denhardt's solution, 0.1% SDS at 20°C for 30 minutes and 90 minutes respectively. After prehybridisation in excess buffer (0.1 ml/cm²), the prehybridisation solution was changed for fresh buffer (0.02 ml/cm²) containing labelled probe. For the hybridisation 0.25 × 10⁶ cpm of ASO was added per ml of hybridisation buffer. The filters were washed for 15 minutes three times in 2 × SSC/0.1% SDS at room temperature and then exposed to Amersham Hyperfilm MP for two days.

Results

We have tried a variety of labour saving approaches to achieve throughput sufficient to undertake molecular genetic studies of the LDLR gene (and other genes) in hundreds of subjects and families with a clinical diagnosis of familial hypercholesterolaemia. Several of

Table 1 Utilities for improvement of throughput for SSCP

Modification	Improvements	Space saving	Staff time saving	Cost saving
96 well arrays	(1) Pre-PCR template replicas readily prepared (2) Minimal recording of location of samples		10-fold Estimated to save 2 hours per 100 samples	
DNA templates dried pre-PCR in 96 well PCR plates	(1) Easy to set up PCR, only have to add reaction mix. No sample volume to take into account (2) Can be stored at room temperature, no requirement for fridge or freezer (3) No labelling of tubes	4-fold compared with same number of samples in 1 ml tubes in standard box		
Double gels (two gels between three glass plates)	Reduces number of apparatus needed	2-fold	See above	2-fold
Repeat loading of gels	(1) Makes maximum use of gels and apparatus (2) Improves throughput	2-fold		2-fold
4.5 mm pitch sharktooth combs	(1) Improves throughput of samples through compatibility with multichannel pipettes		2-fold 8- or 12-fold	
10-fold reduction of unlabelled dCTP and [α - 32 P]dCTP in PCR reaction	(1) Reduces exposure of staff 10-fold (2) Reduces cost			10-fold

these have proven to be of high utility (table 1) and in combination will increase productivity for such studies by an order of magnitude. The autoradiograph of an SSCP gel loaded by repetitive loading with two sets of samples is shown in fig 1; SSCP variants are present in both first and second loadings, and the single and double strands of both loadings can be clearly distinguished. Thirty-two SSCP variants were identified among 791 patients with a PCR failure rate of 5%. These patterns were confirmed by repeat analysis from the original DNA preparations and the results of this analysis are shown in fig 2. A patient with a mutation described previously in exon 3 of the LDLR gene, E80K, was included in the current study. Although it appears that there is at least one other variation in exon 3 displaying a similar SSCP profile to this patient, we tentatively identified several other possible patients with

the same mutation. To confirm this, DNA from all people with an SSCP variation was amplified and tested with allele specific oligonucleotides, as shown in fig 3, to prove the identity of the SSCP profile apparently characteristic of the E80K mutation. This mutation was identified in 11/791 (1.4%) patients and accounted for a third of the patients with an SSCP variation in exon 3. The remaining 21 SSCP variants have not yet been characterised.

Discussion

The large increase in the number of papers citing SSCP as their method of mutation

Figure 1 Autoradiograph of a double loaded SSCP gel (see Methods). The single and double strands of the first and second loadings are designated respectively on the right and left, SS1, SS2, and DS, the double strands having migrated approximately 30 cm and the single strands approximately 20 cm in the gel. SSCP variants are apparent in one sample from the first loading and in one from the second loading, as indicated.

Figure 2 Confirmatory SSCP analysis of PCR products from LDLR gene exon 3 in familial hypercholesterolaemia patients, showing different patterns of variation in exon 3. There are four single strand (ss) bands (presumed to represent multiple conformations), and one double strand (ds) band. In heterozygotes for LDLR gene exon 3 mutations, additional single strand bands are evident. For example, the pattern common to tracks 4, 5, 8, 10, and 15 represents the E80K mutation.

Figure 3 Allele specific oligonucleotide analysis for the E80K mutation in patients with SSCP variations in LDLR gene exon 3. Conditions were as described under methods. PCR products at positions 1A, 1E, 1G, 2D, 2E, 2H, and 3A contain one normal allele and one E80K allele. Row 5 contains positive controls (with one E80K allele). Row 7 contains negative controls (no E80K allele). Rows 4 and 6 are blank.

screening for putative but unknown mutations in segments of DNA, compared with the number citing techniques such as denaturing gradient gel electrophoresis,²⁰ chemical cleavage of mismatch,²¹ and temperature gradient gel electrophoresis,²² appears to reflect the relative simplicity of SSCP compared with the other methods in terms of setup or number of steps. Although not sensitive to 100% of nucleotide variations in a sequence, the high efficiency which appears to be achievable with SSCP¹⁵ has made it a powerful preliminary method for research studies undertaking de novo characterisation of mutations. However, many SSCP mobility variations are small (1 to 5%)¹⁹ and long gels (for example, 300 mm) may therefore be advisable for maximum sensitivity for mobility shifts, in conjunction with a high quality resolving matrix. We estimate that the smallest mobility change detectable in exon 3 would be about 1 mm (0.5%). For large numbers of analyses, for example if the technique were to be used more extensively in the diagnostic setting, labour saving modifications would be essential. We have described here (Methods and table 1), a series of utilities which can increase throughput by an order of magnitude or reduce the time for sample handling, and which have not reduced sensitivity of detection of SSCP variants, as judged from the detection of positive controls.

The use of 96 well microtitre arrays and compatible multichannel devices are well established for cell culture and for analytical reactions. We have recently described a system for simple pattern recognition analysis of known mutations by PCR using horizontal polyacrylamide gels, in which the wells retain the 96 well array (microtitre array diagonal gel electrophoresis, MADGE)²³⁻²⁵ (patent application GB9324901.9). The approach described here also uses the 9 mm pitch of microtitre arrays for high resolution analysis by SSCP. We have stored all of our DNA preparations in master 96 well arrays from which many replicas can be readily made into 96 well plates for PCR. There is no labelling of tubes and the plate is also the storage rack (with identity of sample being related to its position in the array). The storage of many

such plates, each containing a small volume of pre-PCR template in fridges or freezers is inconvenient and expensive. To overcome this problem, we allow the DNA templates to dry overnight at room temperature and store all such plates at room temperature. There are the additional advantages that because the template DNA is dry, it has no volume to be taken into account when setting up the PCR reaction and it also reduces the probability of cross contamination when using an automatic multi-pipette to aliquot the PCR mix. Several wells are left empty of DNA in each array, so that cross contamination can be monitored and so that control samples can be added when necessary. In several months using this procedure we have not found any cross contamination. For samples from which repeated and different PCRs will be undertaken, this enormously reduces necessary staff time and reduces any requirement for laboratory equipment for storage.

"Double gels" and "repeat loading" will each effectively halve the number of gel apparatus, power packs, space, and staff time required to set up analyses of a given number of PCR products. This represents a four-fold improvement in rate of SSCP analysis of PCR products, or four-fold economy in equipment and time. Published approaches for throughput enhancement include pooling of template DNA samples before analysis,²⁶ pre-PCR multiplexing of different pairs of PCR primers,²⁷ post-PCR ethanol co-precipitation of products before electrophoretic and blot analysis,²⁸ and the use of different detection moieties for independent PCR products electrophoresed on the same tracks.²⁹ For SSCP, the detection of variant bands in a pool of product from different templates would be difficult and equalisation of competition between templates pooled pre-PCR is problematical. Some multiplexing of PCR primer pairs may, however, be possible. Post-PCR ethanol co-precipitation appears to be worthwhile if many (for example, more than 10) PCR products are being handled, although subsequent differential probing of blots, or different reporter groups is then necessary. Repetitive gel loading has the advantage that co-precipitation is not necessary

LDLR gene, whereas the G to A transitions change critical glutamates to lysines. Studies of the male germline suggest global methylation in the human LDLR gene (I D Cash, I N M Day, unpublished data). It is likely that there is mutational drive owing to methylation at these CpG sites, and therefore it can be expected that there will be both founder effects (minor or major) because mutations will have occurred early in human evolution (apparently with low negative selection bias³⁴) and recurrent mutations at the same sites because they are estimated to be 20-fold more susceptible to mutation than other dinucleotides,³² mutating at an estimated rate of 10^{-7} per site per gamete.³⁵ Construction of simple diagnostic assays for mutations at these sites should therefore prove worthwhile.

At present, we do not have complete data on the sequence changes causing the exon 3 SSCPs or the effects of these changes at the cellular level on plasma lipid levels or clinical phenotype. The main purpose of this paper is to describe a number of modifications of the SSCP method to improve throughput for mutation screening. The availability of the techniques will enable large groups of patients to be screened for such genotype-phenotype studies and also for diagnostic studies.

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