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The Comparison of the Effectiveness of a Modified Conformation Sensitive Gel Electrophoresis with Denaturing High Performance Liquid Chromatography

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ABSTRACT

Background: Several methods have been developed for detection of sequence variation in genes and each has its advantages and disadvantages. A disadvantage of them is that the simpler, cost-effective methods are commonly perceived as being less sensitive in their detection of sequence variation, whereas those with proven sensitivity have a requirement for complex or expensive laboratory equipment. In this context, we undertook improvements to the conformation sensitive gel electrophoresis (CSGE) method which provides a cost-effective approach to mutation detection and compared the results with scanning carried out using denaturing high performance liquid chromatography (DHPLC) which utilises a dedicated analyser. **Methods:** We designed PCR primers to amplify the seven protein-coding exons of the human *SPP2* gene which encodes secreted phosphoprotein 24 (spp24) such that the amplified products included the immediately-adjacent intronic regions. Five improvements were made to the CSGE method that was used to the scan the PCR-amplified DNA. The scanning was then repeated using DHPLC and the results were compared. **Results:** Using CSGE, a single nucleotide polymorphism was discovered in exon 2 and another in intron 2 of the gene. Re-scanning of the same regions by DHPLC detected no additional sequence polymorphisms. **Conclusion:** With modification of the original protocol, CSGE is capable of providing a simple and cost-effective approach to the detection of DNA sequence polymorphisms that appears to be comparable in sensitivity to DHPLC. *Iran. Biomed. J. 12 (2): 109-114, 2008*

Keywords: PCR, Polymorphism, Single nucleotide polymorphism (SNP), Conformation sensitive gel electrophoresis (CSGE), Denaturing high performance liquid chromatography (DHPLC)

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INTRODUCTION

The ability to detect single-base changes (mutation detection) is of fundamental importance in molecular genetics. This is especially true in human genetics, where it is crucial to identify causative mutations in genes identified to give rise to particular inherited diseases. For the great majority of the diseases, there is extensive allele heterogeneity, and genetic testing may require a search for mutations anywhere within or near the relevant gene or genes. A major issue in laboratory genetic diagnosis is the identification of a quick, inexpensive and reliable method for mutation scanning. Several different methods have been developed and they have their advantages and disadvantages from the point of view of an individual researcher or a diagnostic laboratory. DNA sequencing, single-strand conformation polymorphism (SSCP) and heteroduplex analysis have been the methods most frequently used. Nowadays, the vast majority of the scanning methods attempt to identify new sequence polymorphisms or mutations based on heteroduplex formation between a single strand of the DNA being examined and the complementary strand of DNA that does not have any mutation [1-3].

Conformation sensitive gel electrophoresis (CSGE) was developed as a heteroduplex-based mutation detection system that could be implemented at low cost and with relatively simple laboratory equipment [4]. CSGE exploits the ability

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of solvents such as ethylene glycol and formamide to induce subtle conformation changes in PCR-amplified DNA such that hetroduplexes are differentiated from homoduplexes by their altered electrophoretic migration. Although the method has undergone development to improve its sensitivity [5, 6] it has not been widely adopted, perhaps because of the perception that it might not be as sensitive as other mutation-detection methods such as denaturing high performance liquid chromatography (DHPLC) [7]. Different parameters (including temperatures, running times, the lengths and thickness of the gel, polyacrylamide concentration, manual or fluorescent staining, voltage etc.) have great impacts on the sensitivity and specificity of the method [8-10].

In one of our studies in order to determine the possible function of the human *SPP2* gene, we tried to identify the presence of any polymorphism (SNP) in the coding regions of the human *SPP2* gene. To achieve this goal, we compared the ability of CSGE and DHPLC to scan genes for sequence mutation in the context of the human *SPP2* gene which encodes secreted phosphoprotein 24 (spp24), a non-collagenous extra-cellular matrix protein [11].

MATERIALS AND METHODS

Genomic DNA purification. Human genomic DNA had previously been prepared by various standard protocols from anonymous blood samples that had been taken with informed consent from European individuals.

Primer design and PCR amplification. PCR primers were designed with the computer program Primer3 [12] using DNA sequence data for the *SPP2* gene (accession number AJ272265). All PCR were set up in a 10-µl reaction volume in 200-µl tubes or 96-well micro titre plates and cycling was carried out in a PTC-200 DNA Engine Peltier thermal cycler (MJ Research, USA). In each reaction, 1 mM of each dNTP, about 20 ng template DNA and an optimised amount of primer (0.5 µM) were used in the PCR buffer (Tris-HCl, pH 8.8, 45 mM; ammonium sulphate, 11 mM; 2-mercaptoethanol, 6.7 mM; EDTA, pH 8.8 and 4.4 µM and BSA 113 µg/ml) described by Jeffreys *et al.* [13] with 0.5 units Taq DNA polymerase (ABgene, UK). PCR optimisation was achieved by varying the annealing temperature between 55°C and 65°C in 1°C steps, and by using PCR buffer with $MgCl_2$ concentrations of 1.0-4.5 mM in 0.5 mM steps. The PCR conditions for each fragment including the sequence of each primer, the sizes of fragments produced by amplification, the optimised annealing temperature and $MgCl_2$ concentration are shown in Table 1. The standard cycling conditions used were 30 cycles, each comprising 96°C for 30 s, annealing temperature for 30 s, 72°C for 30 s.

Multiplex PCR. This method was used for co-amplification of multiple exons (4, 5, 6 and 7). By designing appropriate primers (Table 1) with similar annealing temperature (59°C) and optimized $MgCl_2$ concentrations (4 mM) that would amplify products with reasonable length differences, exons 4, 5, 6 and 7 were amplified simultaneously. The concentration

Table 1. The human *SPP2* primers. The sequences of the primers, the sizes of fragments generated by PCR, the concentration of magnesium ions in each PCR reaction, and the annealing temperature (Ann. T.).

Exon(s)	Primers 5′→3′	Size of PCR fragment	$[Mg^{+2}]$ (mM)	Ann. T.°C
1+2	Forward: CAGAAATATTGACCCCAGGA Reverse: GACAGCATTGGAAGGAGGAG	511 bp	4.5	61
2	Forward: CTGCTCTGGATCATGCAGAG Reverse: GACAGCATTGGAAGGAGGAG	245 bp	4.5	63
3	Forward: GCTTTCATGGTGGACAATTC Reverse: CATTTCTGGGATGGGTCTC	268 bp	3.5	60
4	Forward: CAATGGAGGCTATCCCTTTCC Reverse: CCTAAGAGGTGGGGTCTGG	217 bp	4	59
5	Forward: TTTCATGTGCTGACACATCC Reverse: AATGACTCACTAACAAAGAGTTGC	173 bp	4	59
6	Forward: AACATTCTGGAACAGTGAGAGG Reverse: TGATCAGAAAAGGGTCTGGTG	153 bp	4	59
7	Forward: AGAGCCTATGCTTCCCTTTTC Reverse: CAGCAGTTTTAAGGCGTTCAC	199 bp	4	59

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of 0.5 μM for each primer, 2 mM for different dNTP and 0.5 unit Taq DNA polymerase were used. The PCR reaction buffer composition was similar to the single PCR method but BSA was omitted from PCR reactions whose products were to be analysed directly by DHPLC.

Restriction enzyme digestion. Digestion of DNA samples with restriction enzymes (*Fok*I, *Hha*I and *Bsm*AI) was carried out according to the manufacturer's recommendations (New England Biolabs, USA).

Agarose gel electrophoresis. Agarose gels of various concentrations were prepared in 1× TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA) electrophoresis buffer. Electrophoresis was carried out at 100 V, constant voltage.

CSGE. CSGE method was carried out as described by Körkkö *et al*. [5], but with the following modifications: 1) the gel thickness was reduced to 0.36 mm; 2) the gel matrix comprised polyacrylamide: piperazine diacrylamide, (PDA; Bio-Rad, USA) 99:1; 3) a 6× sucrose-based loading dye [sucrose 40% (w/v), xylene cyanol FF 0.25% (w/v), bromophenol blue 0.25% (w/v)]; 4) electrophoresis was carried out at constant voltage (1300-1500 V) instead of constant power; 5) Two concentrations of polyacrylamide gel (20 and 15%) were used, depending on the size of the analysed fragments.

Electrophoresis was carried out on 40 cm × 30 cm gels in a Model S2 sequencing gel electrophoresis apparatus (GIBCO-BRL: Life Technologies, USA). Following electrophoresis, the gel plates were separated, leaving the gel attached to one plate. The gel was then stained by carefully pouring a solution of 1 $\mu g.ml^{-1}$ ethidium bromide on to the surface of the gel where it was left for 2 min. DNA bands were then visualised with a hand-held UV lamp in a darkened room. The relevant portions of the gel were cut from the gel by scalpel and transferred, by blotting, on to a piece of Whatman 3MM paper and stained again with ethidium bromide for 10 min. After de-staining in distilled water for 10 min, the gel fragments were released onto the surface of a UV transilluminator by flooding the Whatman 3MM paper with water. Finally, the DNA bands were visualised by UV illumination and photographed.

DNA sequencing. PCR-amplified DNA was purified by agarose electrophoresis onto dialysis membrane. Purified DNA eluted from the membrane was sequenced using BigDye Termintor v3.1 Terminator Cycle Sequencing reagents (Applied Biosystems, USA) and one PCR primer as the sequencing primer. Electrophoresis was carried out on an ABI PRISM 377 sequencer (Applied Biosystems).

DHPLC. All procedures were carried out according the manufacturer's recommendations (Transgenomic WAVE DNA Fragment Analysis System, USA). The optimal melting temperature for partial denaturation on the DHPLC apparatus was determined for each PCR product by varying the melting temperature in the range 6°C below and above the computer-predicted temperature in 1°C steps.

RESULTS

PCR amplification of the protein-coding exons of the human SPP2 gene. The human *SPP2* gene comprises 8 exons of which the final exon encodes entirely 3´ untranslated region of the mature mRNA. Hence, only the first seven, protein-coding, exons were studied. The intron separating exons 1 and 2 is only 99 bp allowing exons 1 and 2 to be conveniently co-amplified in a single PCR product. Exons 1 + 2, and 2 to 7 were PCR amplified individually, but exons 4, 5, 6 and 7 were also co-amplified in a multiplex PCR reaction. Figure 1 shows an agarose gel of the PCR products from optimised reactions for exons 1-7 of the human *SPP2* gene.

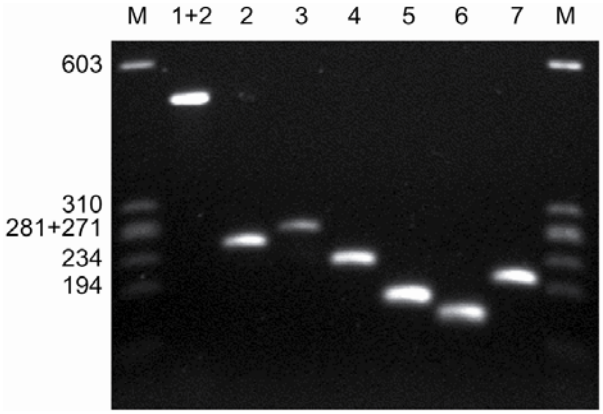

Fig. 1. PCR amplification products of the human *SPP2* exons following agarose gel electrophoresis. The lane designations indicate the amplified exons. M, marker (ΦX174RF/*Hae*III). The sizes of the markers are indicated in bp.

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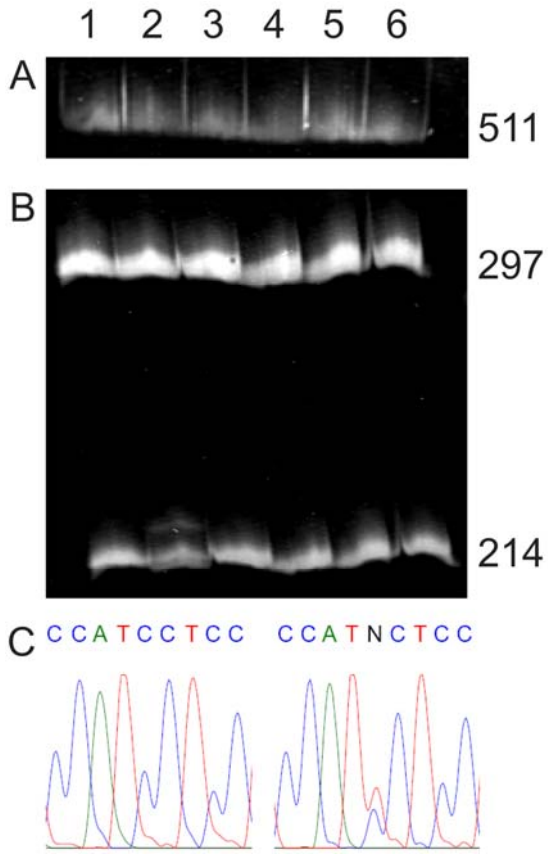

Fig. 2. CSGE detection of a sequence variation in exon 2. (A), CSGE analysis of exons 1 + 2 (511 bp) for six samples; (B), CSGE analysis of the same PCR products following digestion with *Hha*I (297 and 214 bp). The sample in lane 2 exhibits extra faint bands both above and below the exon-2 band (214 bp); (C), DNA sequencing traces for a normal control (left) and for the sample in lane 2 (right) which is heterozygous for the polymorphic base (N).

Scanning for sequence polymorphisms in the SPP2 gene. Samples of DNA from 75 unrelated normal individuals were initially PCR amplified in either monoplex or multiplex reactions and the products subjected to analysis by CSGE. Band shifts indicative of sequence polymorphisms were detected for the products from exons 2 and 3.

Exon 2 polymorphism. The combined PCR product for exons 1 + 2 (511 bp) was scanned by the CSGE method, but no heteroduplexes were found (Fig. 2A). This was possibly due to the failure to detect SNP rather than to their true absence, since it has been reported that the efficiency of detection of SNP reduces with larger fragment size [5]. To attempt to overcome this possible limitation, the amplified DNA fragments were digested by the restriction enzyme *Hha*I into two smaller fragments (297 and 214 bp) and scanned again. A heteroduplex band shift was seen in the smaller fragment (214 bp), corresponding to exon 2, in one of the samples (Fig. 2B).

The band shift was confirmed using primers specific for exon 2 alone (data not shown) and DNA was sequenced from individuals with and without the band shift (Fig. 2C). In the polymorphic allele, base 102 from the beginning of the PCR product was changed from C to T resulting in a change of amino acid from serine to phenylalanine at position 38 of the human spp24 protein. The possible functional significance of this amino acid change is beyond the scope of this paper but has been discussed previously [7].

To enable the rapid screening of larger numbers of samples, it was determined that the polymorphic allele could be detected as a restriction fragment length polymorphism (RFLP) through the loss of a *Fok*I restriction enzyme site relative to the normal allele. Three individuals heterozygous for the polymorphism, and no homozygotes, were identified in a total of 285 samples that were screened by restriction enzyme digestion. Therefore, the allele frequency is about 0.0053 in the population and there is an expectation of approximately 1 person in 36,000 being homozygous for the allele if the sampled population is in Hardy-Weinberg equilibrium.

Intron 2 polymorphism. Scanning by CSGE yielded one common heteroduplex band shift in the exon-3 PCR product (Fig. 3) which DNA sequencing revealed to be due to a G to A base change at the 46th base of the 268-bp PCR amplified fragment (data not shown). This places the polymorphism in intron 2 of the gene, 40 bp upstream of the start of exon 3. This SNP has been identified independently and has been assigned the dbSNP identifier rs17865708.

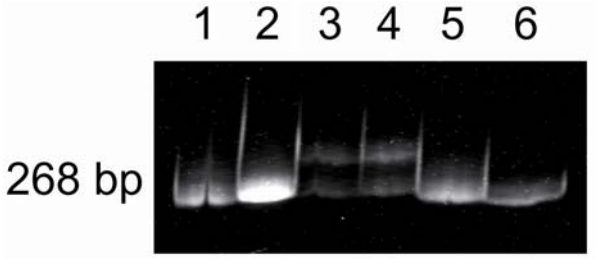

Fig. 3. CSGE scanning of exon 3. Lanes 3 and 4 show the presence of a mismatch.

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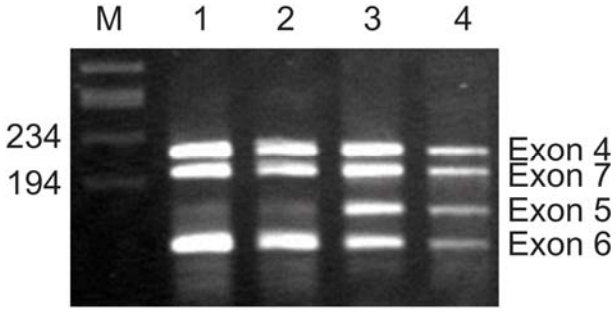

Fig. 4. Multiplex PCR of exons 4 (217 bp), 5 (199 bp), 6 (177 bp) and 7 (153 bp). Exon 5 has amplified less efficiently in samples 1 and 2 compared with samples 3 and 4. M, marker (ΦX174RF/*Hae*III). The sizes of the markers are indicated in bp.

The polymorphic allele gains a recognition site for the restriction enzyme *Bsm*AI relative to the normal allele. To determine the frequency of the polymorphic allele, 138 DNA samples from unrelated normal individuals were PCR amplified and digested by *Bsm*AI. The frequency of the polymorphic allele was about 0.073 in the normal population and the RFLP method confirmed the results of CSGE.

Scanning for variation in exons 4, 5, 6 and 7. The PCR primers for exons 4, 5, 6 and 7 were designed to allow the multiplex amplification of these four exons and their simultaneous scanning by CSGE. Samples of DNA from 144 unrelated individuals were amplified by multiplex PCR, but exon 5 was amplified with variable efficiency from sample to sample (Fig. 4) even after optimising the Mg^{2+} ion concentration and the annealing temperature. Because of this, exon 5 was also amplified independently. Using the CSGE method, no heteroduplexes or mismatches was found in these samples.

Comparison of CSGE to DHPLC. Since only two polymorphisms were detected during the analysis of the protein-coding exons of *SPP2* (one in exon 2 and one in intron 2), it is possible that more SNP existed but were not detected by CSGE. Therefore all samples previously analysed by CSGE were re-analysed by DHPLC. The optimal melting temperatures for partial denaturation on the DHPLC apparatus were as follow: exons 1 + 2, 59°C; exon 2, 56°C; exon 3, 56°C; exon 4, 58°C; exon 5, 54°C; exon 6, 57°C; exon 7, 57°C. The analysis by DHPLC did not reveal any additional SNP.

DISCUSSION

The process of choosing a mutation scanning method is determined by several considerations. Ideally, the chosen method will be specific, sensitive and cost effective, not just in running costs, but also with respect to capital considerations. In addition, freedom from chemical and radiochemical hazards ranks as an important consideration. Amongst the available methods, DHPLC is widely considered to meet the criteria of safety, specificity and sensitivity, but the initial capital cost for the purchase of a DHPLC system remains a barrier for smaller research and service laboratories. By comparison, CSGE is a method that does not require expensive equipment and for which the running costs are relatively low. However, it does suffer from the perception that it is perhaps not capable of detection all sequence polymorphisms [14] but this is probably a consequence of failure to fully optimise the conditions for mutation detection.

We undertook a re-assessment of CSGE and introduced five changes that improved the sensitivity of mutation detection relative to the previously-standard protocol [5]. The thickness of the gel was reduced to 0.36 mm to yield an improvement in electrophoretic resolution, but this also necessitated using PDA as the cross linker to improve the mechanical strength of the gel. The use of a sucrose-based loading dye also improved band resolution as did electrophoresis at constant voltage, though this did result in gel-heating issues that had to be addressed by the use of an electrophoresis apparatus with an integral metal plate to dissipate excess heat. The final improvement was to run higher percentage gels and to adapt the gel concentration to the length of the DNA fragments being analysed.

With the incorporation of a few simple modifications, we have improved the CSGE method such that DHPLC was unable to detect any additional sequence SNP in the samples that were analysed. However, we and others have noted that CSGE can miss detecting DNA sequence polymorphisms that lie close to the ends of PCR-amplified fragments or in fragments that are too large. In screening the exons of the *SPP2* gene, we addressed the first issue by ensuring that the PCR primers were placed sufficiently far enough into the adjacent introns. Indeed, this approach led to the successful detection of the SNP in intron 3 of the gene. The second issue requires that the PCR-amplified fragments not be too large or that the products be cut with restriction enzymes prior to

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electrophoresis as we did with the exon 1+2 fragment in which we detected the exon-2 polymorphism. We have previously adopted this restriction-enzyme-digestion approach as an improvement to the SSCP mutation detection method [15], though further investigation of its general applicability to CSGE is required.

Based on this study the advantages of CSGE include: 1) It is very simple and does not need any complex equipment or facilities; 2) It requires no special preparation of the PCR product; 3) It can handle a large number of samples; 4) It can simultaneously scan several DNA fragments from multiplex PCR; 5) It does not require the use of radioactive substances; 6) It is inexpensive and its interpretation is very simple. The limitations of CSGE include: 1) It cannot reveal mismatches which fall on the flanking regions of fragments larger than 400-500 bp (like other heteroduplexes analysis methods). This limitation can be overcome using restriction digestion to cut the PCR products into smaller fragments prior to analysis; 2) It does not reveal the position of any base change.

In summary, CSGE represent a viable approach for the reliable detection of DNA-sequence variants in a cost-effective manner with the use of relatively simple laboratory equipment.

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