Brazilian Journal of Medical and Biological Research (1999) 32: 55-58
ISSN 0100-879X Short Communication

Detection of point mutations by non-isotopic single strand conformation polymorphism

N.A. Pinheiro[1,2], R.P. Moura[2], E. Monteiro[3] and L.L. Villa[2]

[1]Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, São Paulo, SP, Brasil
[2]Instituto Ludwig de Pesquisa sobre o Câncer, São Paulo, SP, Brasil
[3]Hospital do Câncer, Fundação Antonio Prudente, São Paulo, SP, Brasil

Abstract

We have developed a procedure for nonradioactive single strand conformation polymorphism analysis and applied it to the detection of point mutations in the human tumor suppressor gene p53. The protocol does not require any particular facilities or equipment, such as radioactive handling, large gel units for sequencing, or a semiautomated electrophoresis system. This technique consists of amplification of DNA fragments by PCR with specific oligonucleotide primers, denaturation, and electrophoresis on small neutral polyacrylamide gels, followed by silver staining. The sensitivity of this procedure is comparable to other described techniques and the method is easy to perform and applicable to a variety of tissue specimens.

Key words
- p53 Gene
- Human papillomavirus
- PCR
- SSCP
- Cancer
- Silver staining

Correspondence
L.L. Villa
Instituto Ludwig de Pesquisa sobre o Câncer
R. Prof. Antonio Prudente, 109
4º andar
01509-010 São Paulo, SP
Brasil
E-mail: ludwig2@eu.ansp.br

Publication supported by FAPESP. N.A. Pinheiro was the recipient of a CNPq fellowship.

Received July 7, 1998
Accepted November 3, 1998

A variety of methods have been proposed that facilitate rapid identification of genetic polymorphisms or somatic cell mutations, among which single strand conformation polymorphism (SSCP) is probably the most widely used. The original SSCP protocol uses radiolabeled oligonucleotides to generate a radioactive PCR product, which is then highly diluted, heat denatured, and analyzed by electrophoresis in a large (40 x 20 cm) nondenaturing gel (1). SSCP is based on the differential sequence-dependent electrophoretic mobilities of single-stranded DNA in nondenaturing polyacrylamide gels: changes in the sequence can cause a shift in the mobility of the analyzed conformers. This method, although sensitive, is both time consuming and cumbersome. There are several reports describing non-isotopic protocols for SSCP analysis (2,3), but these require expensive reagents and equipment such as small-format PhastGel, PhastSystem and Hydrolink-MDE, or the use of composite agarose gels (4).

We developed a protocol for the detection of point mutations and deletions by a non-isotopic SSCP analysis on standard silver-stained polyacrylamide gels. The sequences used were PCR amplified regions of the p53 gene from samples of cervical carcinomas, penile carcinomas and gastric carcinomas where point mutations had been previously identified by isotopic SSCP and sequencing.

PCR products were generated by specific primers and conditions for exons 5, 6, 7, 8 and 9 of the p53 gene, using the following primers: for exon 5, 5'-TACTCCCCTGCC

CTCAACAAG-3' and 5'-CACCATCGCTA TCTGAGCAGCG-3'; exon 6, 5'-CAGGG CTGGTTTCCCAGGGTCCCCA-3' and 5'-CAGGCGGCTCATAGGGCA-3'; exon 7, 5'-GTGTTATCTCCTAGGTTGGC-3' and 5'-CAAGTGGCTCCTGACCTGGA-3'; exons 8 and 9, 5'-CCTTACTGCCTCTTGC TTC-3' and 5'-CTGGAAACTTTCCAC TTGAT-3'. Reactions were performed in a final volume of 25 µl containing 0.2 mM of 4 deoxyribonucleotide triphosphates, 10 mM Tris-HCl, pH 8.0, 50 mM KCl and 1.5 mM $MgCl_2$, 0.5 unit Taq DNA polymerase (Cenbiot, Porto Alegre, RS, Brazil), 1 µM of each primer and the following cycling profiles: for exon 5, after heating for 5 min at 93°C, 35 cycles of 5 min at 93°C, 30 s at 58°C, and 2 min at 72°C; for exons 6 and 7, 1 cycle at 94°C for 5 min, 32 cycles of 5 min at 94°C, 1 min at 63°C, and one cycle at 72°C for 7 min; for exons 8 and 9, 1 cycle at 95°C, 35 cycles of 1 min at 59°C, 1 min at 72°C, 1 min at 95°C, and 1 cycle of 2 min at 59°C, 5 min at 72°C. Following amplifications, 8 µl of the PCR product was mixed with 4 µl of 95% formamide containing 0.05% xylene cyanol, 0.05% bromophenol blue and 20 mM EDTA, heated at 95°C for 10 min and loaded onto a 1 cm x 19 cm x 20 cm 7.5% polyacrylamide: bis-acrylamide (49:1) gel containing 5% glycerol. Electrophoresis was performed at room temperature for 5 h at 30-mA constant current or until the bromophenol blue dye reached the bottom of the gel, which was then stained with silver (5).

All the mutations detected using radioactive SSCP conditions where also detectable using the non-radioactive methodology and standard gel format described here (data not shown). A list with some of the mutations detected is shown in Table 1. Examples of typical analyses are shown in Figure 1.

A common problem in assessing gene mutations is that the tissue specimen being analyzed is often heterogeneous and may contain cells bearing the normal counterpart of the gene under investigation. Therefore, the results may be obscured if a large proportion of normal tissue is present in the tumor sample. We have investigated this possibility by applying the described protocol to artificial mixtures of normal and mutated exon 6 of the p53 gene, cloned into pUC18. As shown in Figure 2, an altered SSCP profile is still observed when as little as 5% of the mutated sequence is present.

The present methodology reduces processing time, exposure, and biohazard, and shows no reduction in sensitivity when compared to other methods including radioac-

Table 1 - p53 Gene mutations in human tumors.

Exons amplified from carcinomas of the uterine cervix (a), stomach (b), and head and neck (c).

Exon	Codon	Nucleotide changes	Alteration
5[a]	175	substitution GCG/GCA	
5[c]	138	substitution GCC/GTC	ALA/VAL
5[c]	157/158	insertion (+4)	Frame shift
5[c]	155 to 158	deletion (-12)	THR-ARG-VAL-ARG
6[b]	191	deletion (-3)	PRO
6[a]	213	substitution GAC/GGC	ASP/GLY
7[c]	249	substitution AGG/TGG	ARG/TRP
8[a]	277	substitution ACA/AAA	THR/LYS
8[c]	269 to 285	deletion (-47)	Frame shift
8[a]	278	substitution CCT/CTT	PRO/LEU
9[c]	308	deletion (-1)	Frame shift
9[c]	314 to 315	deletion (-5) TCCTC	Frame shift

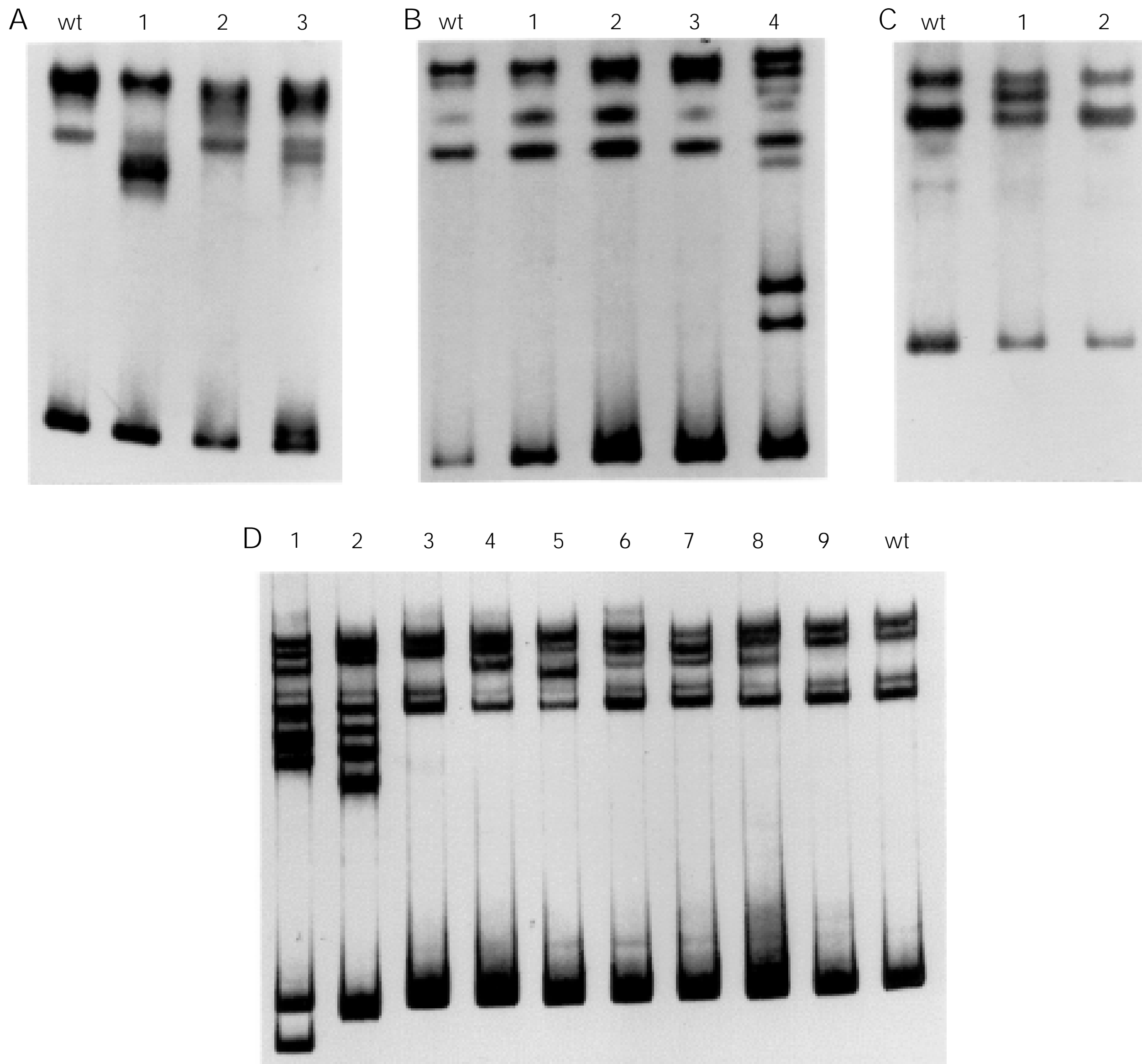

Figure 1 - p53 Gene alterations detected by non-isotopic SSCP in standard silver-stained polyacrylamide gels. A, PCR products of exon 5 amplified from an HPV-positive cervical carcinoma with a single nucleotide substitution (G→A) (lane 1), an HPV-negative cervical carcinoma with a normal exon 5 sequence (lane 2), a gastric carcinoma with a single nucleotide substitution (G→A) (lane 3). B, PCR products of exon 6 from HPV-positive cervical carcinomas with single nucleotide substitutions (A→G) (lanes 1, 2), an HPV-negative penile carcinoma with a single substitution (A→G) (lane 3), a gastric carcinoma with a 3 nucleotide deletion in codon 181 (lane 4). C, PCR products of exon 7 from an HPV-negative penile carcinoma with two nucleotide substitutions (C→A and C→T) (lane 1), an HPV-negative cervical carcinoma with a normal exon 7 sequence (lane 2). D, PCR products of exons 8 and 9 from head and neck tumors with normal (lanes 3, 9) and altered nucleotide sequences (lanes 1, 2, 4, 5 6, 7, and 8). wt: Corresponds to the normal sequences amplified from the cervical carcinoma cell line HeLa (A,B,C) and from a head and neck tumor (D).

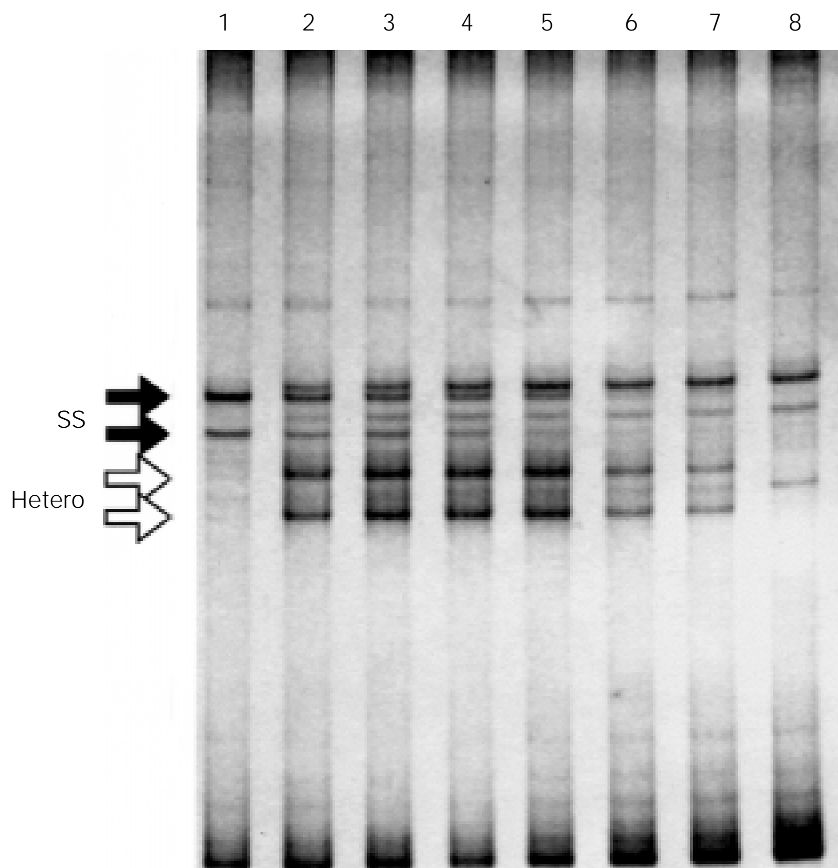

Figure 2 - SSCP-PCR analysis of mixtures of normal and mutated exon 6 of the p53 gene. Different amounts of recombinant pUC18 bearing normal or mutated (9 nucleotide deletion) sequences were submitted to the reaction mix, in a final DNA concentration of 50 ng, either alone (lane 1: mutant; lane 8: normal) or mixed (lane 2: 25 ng each; lane 3: 20 ng mutant, 30 ng normal; lane 4: 15 ng mutant, 35 ng normal; lane 5: 10 ng mutant, 40 ng normal; lane 6: 5 ng mutant, 45 ng normal; lane 7: 2.5 ng mutant, 47.5 ng normal). Arrows indicate the banding pattern of single-stranded (SS) DNA and heteroduplex.

tive-labeling methods, especially when DNA fragments of about 200 bp are analyzed. The use of this method has allowed improved identification of point mutations in samples investigated in other studies currently underway in our laboratory, including both fresh and fixed tissues derived from thyroid tumors (data not shown). Moreover, the procedure is immediately applicable in many laboratories with a relatively modest infrastructure where classical SSCP would not be performed.

Acknowledgments

We are grateful to Dr. Andrew J. Simpson for helpful technical hints and discussions. We acknowledge Roberta U. Bevilacqua for the gastric carcinoma samples and Paula Rahal for the penile carcinoma analysis.

References

1. Orita M, Iwahana H, Kanazawa H & Sekiya T (1989). Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. Proceedings of the National Academy of Sciences, USA, 86: 2766-2770.
2. Ainsworth PJ, Surh LH & Coulter-Mackie MB (1991). Diagnostic single strand conformational polymorphism (SSCP): a simplified non-radioisotopic method as applied to a Tay-Sachs B1 variant. Nucleic Acids Research, 19: 405-406.
3. Oto M, Miyake S & Yuasa Y (1993). Optimization of nonradioisotopic single strand conformation polymorphism analysis with a conventional minislab gel electrophoresis apparatus. Analytical Biochemistry, 213: 19-22.
4. Peng H, Du M, Ji J, Isaacson PG & Pan L (1995). High-resolution SSCP analysis using polyacrylamide agarose composite gel and a background free silver staining method. Biotechniques, 19: 411-414.
5. Sanguinetti CL, Dias Neto E & Simpson AJG (1994). Rapid silver staining and recovery of PCR products separated on polyacrylamide gels. Biotechniques, 17: 915-921.