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Improved Detection of Mutations in the p53 Gene in Human Tumors as Single-stranded Conformation Polymorphs and Double-stranded Heteroduplex DNA

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Research in the last 3 years has made it increasingly clear that a large variety of human tumors contain mutated p53 tumor suppressor genes (reviewed in ref. 1). Since mutations are most often distributed over at least 5 exons (exons 5–9) spanning 2.9 kb of coding sequence, faster methods for scanning the region have been developed.[2]

A popular method for detection of point mutations and deletions in the p53 gene is single-stranded conformation polymorphism (SSCP).[2,3] The technique is based on altered migration speeds through solid supports of single-stranded DNA fragments carrying mutations. The altered migration of DNA is presumably caused by differences in the conformation of single-stranded DNA. The method depends heavily on experimental conditions that optimize migration differences of the conformation polymorphs. Thus, adding glycerol to the polyacrylamide, reducing the temperature, increasing the length of the run, and so forth result in a greater level of reproducibility.[2,3] In spite of these modifications, reproducibility and resolution present recurrent problems using polyacrylamide as a gel support, presenting the need for novel polymers for this purpose. There is also a need to develop a simple nonradioactive technique for screening for mutations in the p53 gene in the clinical laboratory. A gel electrophoresis technique that takes advantage of differences in mobility of wild-type and mutation-bearing DNA fragments could prove useful for this purpose. Double-stranded DNA as heteroduplexes (HTX) migrate at different rates compared to DNA as homoduplexes in solid supports.[4,5] These differences can be visualized easily by staining with ethidium bromide. However, the resolution ability of polyacrylamide gel supports for detection of the two species varies with the sequence of the DNA fragments.[4,5]

In an effort to improve the sensitivity levels of the radioactive SSCP analysis, and to resolve the nonradioactive duplexes, we began to experiment with other polymers. We tested polyacrylamide, Hydrolink-D5000, and Hydrolink-MDE under identical experimental conditions with and without 10% glycerol. We found that Hydrolink-MDE (AT Biochem, Malvern, PA), a vinyl polymer, gave improved resolution of bands as well as reproducibility in the detection of both single-stranded DNA in SSCP and of DNA duplexes in the HTX analyses. Because the SSCP analysis is unable to detect mutations that do not result in polymorphs with altered migrations, a combination of SSCP analysis with HTX analysis might provide more reliable results than either one alone.

Human mammary tumors were analyzed by SSCP for mutations in p53 exons 5–9 using 5% polyacrylamide.[6] A cell line derived from normal breast tissue (HBL-100) was used as a negative control, whereas breast tumor cell lines served as positive controls (SKBR3 and HS578 for exon 5, T47D for exon 6, MW for exon 7, and BT474 for exon 8). The colon cancer cell line, SW480 was used as a positive control for mutation in exon 9 of the p53 gene. It should be noted that these cell lines, carrying mutations in the various exons of the p53 gene, have lost the normal allele of the p53 gene and are therefore, hemi- or homozygous mutants. Primary breast tumor DNAs were also analyzed by SSCP on polyacrylamide and it was found that 10 out of 59 (17%) primary tumors had mutations in this region of the p53 locus.[6] Then, DNA from the same panel of cell lines as well as primary tumors considered to be questionable positives by SSCP analysis on polyacrylamide gels (PGE) was retested with the polymer, Hydrolink-MDE (MDE), a modified polyacrylamide-based vinyl polymer. Representative results are shown in Figure 1 for exon 6 of the p53 gene, as the majority of mutations observed in breast carcinomas were detected in these sequences. It should be noted that the sharp contrast in resolution efficiency seen in PGE versus MDE is reproducible, but is peculiar to exon 6, since the other fragments (exons 5, 7, 8, 9) showed comparable separations in the two gel matrices (shown for exon 8 in Fig. 2). As seen in Figure 1, there is improved resolution of several of the conformation polymorphs in MDE in comparison to PGE. As a result, alterations in migration in the DNA fragments caused by mutations are more easily discernible (compare 4813, 5600, and 4805 in MDE versus PGE). Because single-stranded DNA mobility is also subject to environmental and gel conditions, some mobility differences are questionable with the new polymer (Fig. 1, 5633). Tumors 4811 and 5594 are negative for mutations in exon 6 (Fig. 1), but showed mobility shifts compared to HBL-100 in exons 8 and 9, respectively,

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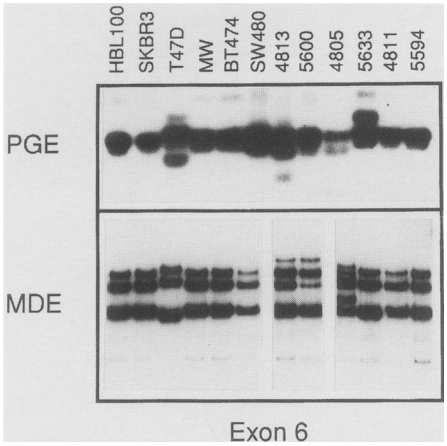

FIGURE 1 SSCP analysis of exon 6 (194 bp) of the p53 gene in breast cell lines and primary breast tumors[6] in a 5% polyacrylamide gel (*upper panel*) versus Hydrolink-MDE polymer (AT Biochem, Malvern, PA; *lower panel*). Three microliters of [α-^{32}P]dCTP-labeled, PCR-amplified, denatured DNA were mixed with an equal volume of sequencing stop solution containing 20 mM NaOH and loaded in these two gel matrices in 1× TBE at 8W for 13–15 hr. The gels were dried and exposed to Kodak XAR for 5 hr at room temperature. HBL-100 cell line is representative of wild-type p53 for exons 5–9, and breast cell-lines SKBR3 (CGC to CAC, codon 175, exon 5), T47D (CTT to TTT, codon 194, exon 6), MW (TAC to TAA, codon 234, exon 7), and BT474 (GAG to AAG, codon 285, exon 8) have known point mutations in exons 5–8,[6] respectively. SW480 is a colon carcinoma cell line with a point mutation (CTC to CCC, codon 309, exon 9) of the p53 gene.[6]

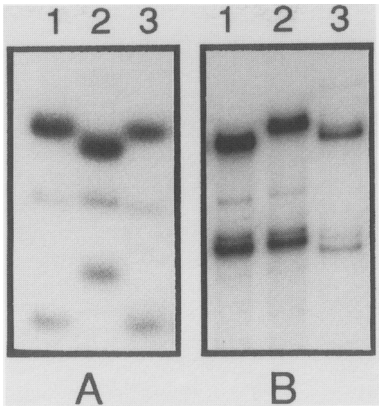

FIGURE 2 SSCP analysis of exon 8 of the p53 gene (209 bp) in polyacrylamide gel (*A*), and Hydrolink-MDE gel (*B*). The samples were loaded as follows: (lane *1*) HBL-100; (lane *2*) BT474 (breast cancer cell line with known mutation in exon 8); (lane *3*) breast tumor 4802.

of the p53 gene.[6] In addition, in three separate exons, we found three new mutations in breast tumor DNA that were previously undetected by analysis of the SSCP products on polyacrylamide gels. The detection of a mutation in exon 8 of the p53 gene of a primary breast tumor by SSCP analysis on MDE (Fig. 2B), undetected by PGE (Fig. 2A), is shown in Figure 2. Unlike exon 6, for fragments of exon 8 of the p53 gene, the resolution of single-stranded DNA is comparable, and the detection of a mutation in DNA of breast cell line BT474 (codon 285, GAG to AAG) is far easier in PGE gels. However, in MDE, the sensitivity of detection has improved enough to suspect a mutation in the tumor DNA (Fig. 2B, lane 3). The presence of a mutation in exon 8 of tumor 4802 was confirmed by nucleotide sequencing.

Point mutations were also visualized by mobility shifts of DNA homoduplexes and heteroduplexes under nonradioactive conditions using ethidium bromide staining. Ten microliters of PCR product of each of exons 5–9 of the p53 gene from the indicated cell lines and tumors were mixed with 2 μl of 6× loading dye [30% glycerol/0.05% bromophenol blue (BPB)/0.05% xylene cyanol (XCFF)/60 mM EDTA], denatured at 95°C for 5 min, then allowed to cool to room temperature for 1 hr to promote random and complete duplex formation. MDE obtained as a 2× concentrate is polymerized using ammonium persulfate (0.04%) and TEMED (0.4%) according to the instructions of the supplier (final: 1× MDE, 0.5× TBE). Samples were then loaded and electrophoresed in a 40-cm × 20-cm gel in 0.5× TBE buffer at 4 watts for 12 hr at room temperature, with a metal plate attached to maintain uniformity of temperature. Under these conditions of electrophoresis, temperature regulation was not found to be necessary. A *Hae*III digest of phage ΦX174 was used as a molecular weight marker. While still attached to the plate, to avoid expansion in water, the gel was stained for 5 min in 1 μg/ml ethidium bromide and then quickly rinsed with water. Duplexes were visualized just below the XCFF dye (approximately 22 cm). Homozygous or hemizygous mutant p53 molecules were expected to migrate as a single band (typified by the cancer cell lines HS578, T47D, MW, BT474, and SW480 in p53 exons 5, 6, 7, 8, and 9, respectively), while PCR products derived from tumor DNA heterozygous at the p53 locus were expected to form up to four bands: mutant/mutant, normal/normal homoduplexes, plus two mutant/normal heteroduplexes. We found migration differences between homoduplexes and heteroduplexes, as well as those between normal and mutant homoduplexes. As seen in Figure 3, migration differences are clearly visible between wild-type p53 homoduplexes

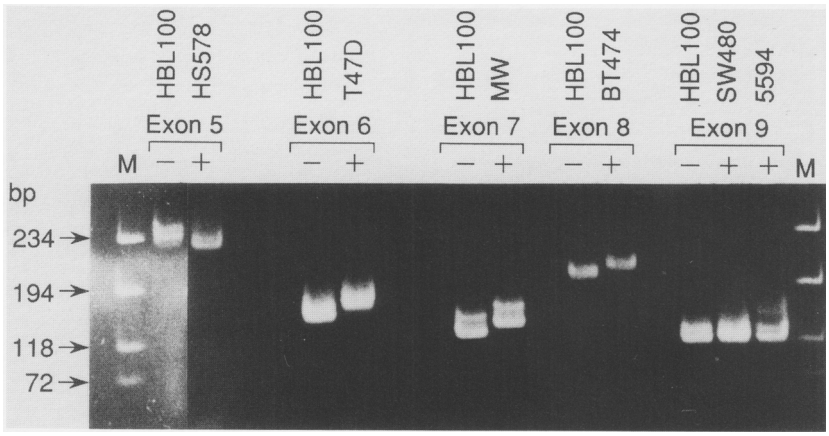

FIGURE 3 Heteroduplex and homoduplex mobility shifts in Hydrolink-MDE polymer of PCR-amplified p53 exons 5–9 in breast cell lines HBL-100, HS578, T47D, MW, BT474, colon carcinoma cell line SW480, and primary breast tumor 5594. Ten microliters of PCR product were loaded with 2 μl of 6× loading dye and electrophoresed in 0.5× TBE buffer at 4W for 12 hr. A *Hae*III digest of bacteriophage ΦX174 was used as molecular weight markers (lanes *M*). The (−) sign indicates wild-type p53, while the (+) sign indicates presence of point mutations in the sequence. The gel was stained for 5 min in 1 μg/ml of ethidium bromide and visualized under ultraviolet light.

formed by HBL-100 (wild-type p53 gene) DNA versus T47D (mutation in exon 6), MW (mutation in exon 7), and BT474 (mutation in exon 8). The differences are more subtle in homoduplexes formed by HS578 (mutation in exon 5) and SW480 (mutation in exon 9). The HTX for the primary breast tumor DNA 5594 (Fig. 3) indicates that it has a mutation in exon 9 of the p53 gene, as previously shown by SSCP analysis with the same polymer. This tumor is heterozygous for mutation in the p53 gene, as deduced from the multiband pattern seen in this assay (Fig. 3, 5594). In our hands, resolution using MDE was clearer and more reproducible to those obtained by using 6% PGE or Hydrolink-D5000 polymers (AT Biochem, Malvern, PA)[4] in the separation of DNA under the conditions described. Using the same size gels, separation on 6% PGE did not resolve the bands. High background staining of the Hydrolink-D5000 gels with ethidium bromide made it difficult to visualize the duplexes. Variations in electrophoretic conditions, such as addition of glycerol and/or urea to the gel, and temperature of fractionation[5] are being tested for further resolution of the HTXs using these matrices.

In conclusion, single base-pair mutations in the p53 gene were detected reproducibly by SSCP analysis in human mammary tumors using a novel polymer, MDE. MDE polymerizes rapidly to a tough membrane, is flexible, and is easy to handle. In addition, use of radioactivity can be eliminated altogether by the analysis of single-base mismatches in PCR-amplified DNA as heteroduplexes using this polymer. Although further work is necessary to optimize its sensitivity, this nonradioactive method of detecting point mutations of primary human tumors shows promise as an inexpensive and practical test in the clinical laboratory. A combination of the two methods, SSCP and HTX, have the potential to reveal mutations not detected by either one of them alone.

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