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A denaturing gradient gel electrophoresis assay for sensitive detection of p53 mutations

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Abstract. p53 is a tumor suppressor gene located on 17p, a region of the human genome frequently deleted in tumors. Mutation of the p53 gene is an important step leading to development of many forms of human cancer. To simplify the analysis of tumors for p53 point mutations, we describe a GC-clamped denaturing gradient gel assay for detecting single-base substitutions within highly conserved regions of the p53 gene. This assay alows for efficient screening of tumors for single-base substitutions within the p53 gene and can be used to facilitate sequence analysis of p53 point mutations.

Introduction

The most common genetic defect found in human cancer cells is mutation of the p53 tumor suppressor gene (Levine et al. 1991). Somatic mutations in this gene have been described in a wide variety of different cancers, including lung, breast, colon, liver, esophageal, ovary, bladder, and prostate carcinomas (Nigro et al. 1989; Levine et al. 1991; Bressac et al. 1991; Hsu et al. 1991; Hollstein et al. 1991). p53 mutations in sarcomas, lymphomas, and leukemias have also been reported (Mulligan et al. 1990; Gaidano et al. 1991; Jonveaux et al. 1991; Fenaux et al. 1991; Toguchida et al. 1992). In addition, germline p53 mutations have been shown to be present in patients with the Li-Fraumeni genetic cancer syndrome (Malkin et al. 1990). Although mutation of the p53 gene can involve deletions and rearrangements, a common form of p53 point mutation is single-base substitution. In fact, over 300 different single-base p53 mutations have been reported (Hollstein et al. 1991). Furthermore, several different germline p53 mutations have been shown to play a role in inherited cancer (Malkin et al. 1990; Metzger et al. 1991), and many more likely exist. Nearly all known p53 single-base substitutions have been shown to occur in exons 5–8, which code for much of the evolutionarily conserved amino acid sequence within the p53 protein (Levine et al. 1991; Hollstein et al. 1991).

The ability to detect p53 mutations efficiently is clinically important for two reasons. First, the detection of germline p53 mutations is important for identifying and counseling patients who are at high risk for developing cancer. Second, efficient detection of p53 mutations in tumor samples is important for studies seeking to determine the clinical significance of p53 mutations. Many p53 mutations have been detected by direct DNA sequencing of polymerase chain reaction (PCR)-amplified p53 sequence. Although this is a sensitive method of identifying single-base substitutions, it is labor intensive. Several techniques have been developed that can be used to screen for mutations within short DNA fragments including RNase cleavage (Myers et al. 1985a), chemical cleavage (Cotton et al. 1988), denaturing gradient gel electrophoresis (DGGE; Fischer and Lerman 1980; Myers et al. 1987; Sheffield et al. 1989), and single-strand conformation polymorphism (SSCP) analysis (Orita et al. 1989a,b). One of the more sensitive techniques is a modification of DGGE, known as GC-clamped DGGE (Sheffield et al. 1989). We have previously utilized GC-clamped DGGE to identify point mutations in the p53 gene in brain tumors (Metzger et al. 1991; Cogen et al. 1992). In this study we present a refined GC-clamped DGGE assay for the p53 gene and demonstrate that this assay is both sensitive and efficient, and can be used in combination with DNA sequencing to identify base substitutions precisely within the p53 coding sequence.

Materials and methods

PCR amplification

Primers flanking p53 exon sequences were selected from p53 genomic sequence and prepared using phosphoramidite chemistry with an Applied Biosystems model 391 oligonucleotide synthesizer. One of each primer pair contained a 5′ 40-bp (base pair) GC-rich sequence (GC-clamp), which becomes incorporated into the final PCR product

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Table 1. Conditions for denaturing gradient gel electrophoresis (DGGE) of p53 mutations

p53 Exon	Primers	Fragment size	DGGE conditions[a]
5	Forward 5'(GC)TTCCTCTTCCTGCAGTACTC-3'[b] Reverse 5'-CTGGGCAACCAGCCCTGTCGT-3'	282	60%–80% for 10.5 h
6	Forward 5'(GC)ACGACAGGGCTGGTTGCCCA-3' Reverse 5'AGTTGCAAACCAGACCTCAG-3'	227	40%–70% for 8 h
7	Forward 5'(GC)TCTCCTAGGTTGGCTCGACTG-3' Reverse 5'GCAAGTGGCTCCTGACCTGGA-3'	174	35%–65% for 6 h
8+9	Forward 5'CCTATCCTGAGTAGTGGTAATC-3' Reverse 5'(GC)CCCAAGACTTAGTACCTGAAG-3'	372	35%–65% for 8 h

[a] % denaturant range and electrophoresis run time at 150 V and 60°C
[b] (GC) is 5'-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCG-3'

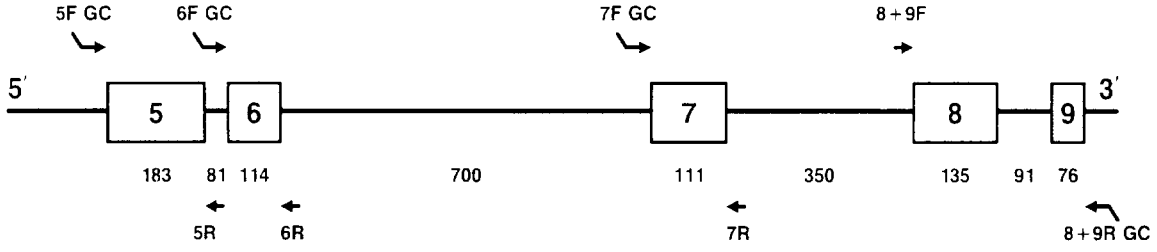

Fig. 1. A map of exons 5–9 of the human p53 gene. Polymerase chain reaction (PCR) primers are indicated by *arrows*. GC-clamped primers are designated by *(GC)*. The sizes in base pairs of exons and introns are indicated by the *numbers* below each gene segment

during the amplification reaction. PCR amplification was performed by using minor modifications of a previously reported protocol (Sheffield et al. 1989). Specifically, 500ng of human genomic DNA or 1 ng of cloned DNA was mixed with 50pmol of each appropriate oligonucleotide primer and with 37.5nmol of each deoxyribonucleotide triphosphate in 100µl of PCR buffer (67m*M* Tris-HCl pH8.8, 6.7m*M* $MgCl_2$, 16m*M* ammonium sulfate, 10m*M* 2-mercaptoethanol, and 10% dimethylsulfoxide). *Thermus aquaticus* DNA polymerase (1.5 units) was added to each sample, and the samples were incubated at 94°C, 55°C, and 72°C for 30s at each temperature in a Cetus thermocycler for a total of 40 cycles. Success of amplification was determined by subjecting 10µl of each amplified sample to electrophoresis on a 1.5% agarose gel.

Mutagenesis

Random mutagenesis was performed on a region of the p53 gene containing exons 5, 6, and 7 using the PCR-based mutagenesis protocol of Zhou et al. (1991).

DGGE

The optimum gradient for each PCR product was determined by analyzing each DNA fragment with perpendicular DGGE as previously described (Myers et al. 1987). In addition, we used the Melt Map computer program (Lerman and Silverstein 1987) kindly provided by Dr. Leonard Lerman to confirm the melting domain profile of each p53 PCR product and to determine the optimal position for the GC-clamp. In addition, travel schedule gels were performed for each fragment to determine the optimum running times by using a previously described protocol (Myers et al. 1987). Samples to be tested for the presence of single-base substitutions were then subjected to electrophoresis on the appropriate parallel denaturing gradient gel. The primer sequences, range of denaturant, and optimum time of electrophoresis at 60°C and 150V for each PCR product are shown in Table 1. Gels were stained with ethidium bromide and were photographed using Polaroid positive/negative film. An exon containing a mutation is recognized by the presence of one or more new bands not present in normal control samples. Multiple bands are the result of heteroduplex formation between a strand of wild-type DNA and the complementary mutant DNA strand during the last annealing step of the PCR process (Sheffield et al. 1989).

DNA sequencing

PCR products were prepared for DNA sequencing by subjecting 90µl of amplified DNA to electrophoresis on a 1.5% agarose gel for 2h at 100V. The amplified product was recovered from the agarose by cutting the appropriate band from the agarose gel, freezing the gel fragment at –70°C for 15min or –20°C overnight, and centrifuging it in a Costar 0.22 Spinex centrifuge tube. Two volumes of ethanol were added to the recovered liquid, and the sample was centrifuged for 5 min in a microfuge. The precipitated DNA was resuspended in 15µl of dH_2O. Sequencing was performed using 7µ of the resuspended sample by automated sequencing using dye terminators and an Applied Biosystems automated sequencer.

Results

The relative position of the PCR primers to the p53 gene are shown in Fig. 1. In each case, the primers flank a highly evolutionarily conserved region of the exon. The conserved regions have been shown to contain the vast majority of p53 mutations (Hollstein et al. 1991). The theoretical melting analysis of each fragment as determined by the computer Melt Map program (Lerman et al. 1987) showed a single melting domain adjacent to the high melting domain introduced by the GC-clamp (Fig. 2).

To determine the utility of the DGGE assay for identifying single-base mutations within the p53 gene we analyzed multiple known mutations. Samples were PCR-amplified using the appropriate primers and analyzed by DGGE. All known mutations tested were detected by this

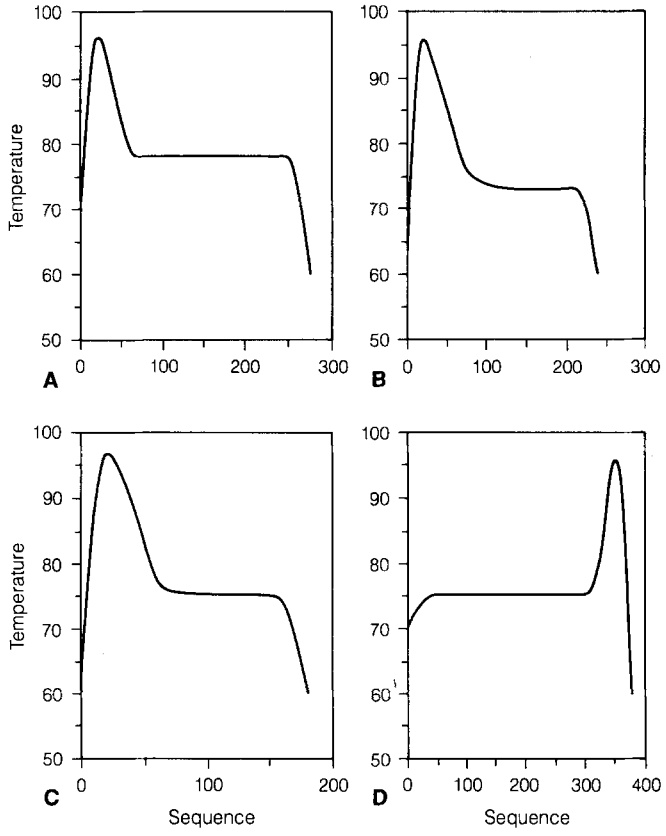

Fig. 2A–D. Theoretical melting profiles as determined by the Melt Map computer program for each of the four PCR-amplified p53 fragments. Each base pair of the sequence is plotted against the temperature (°C) at which the base pair will be 50% in the double-stranded and 50% in the single-stranded state. The GC-clamp sequence is indicated by the peak at 95°C. Note that the p53 sequence (non-GC-clamp sequence) consists of a single melting domain for each of the fragments. The fragments are as follows: exon 5 (**A**), exon 6 (**B**), exon 7 (**C**), and exons 8 and 9 (**D**)

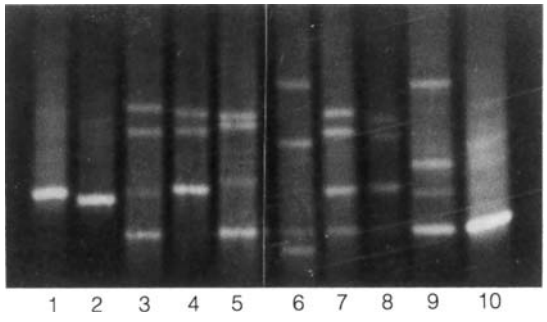

Fig. 3. DGGE analysis of exon 7 of the p53 gene amplified from a variety of human tumors. The DNA in *lane 10* was amplified from normal control genomic DNA. The remaining samples were amplified from genomic DNA isolated from a variety of tumor samples known to contain p53 point mutations. The samples were subjected to electrophoresis on an 8% polyacrylamide gel containing a 35%–60% denaturant gradient for 6h at 150V at 60°C. The presence of shifted bands indicates the presence of single-base substitutions in these samples. Mutations are as follows: GGC to TGC at codon 245, mutant allele only *(lane 1);* CGG to TGG at codon 248, mutant only *(lane 2);* CGG to TGG at codon 248, mutant and normal alleles *(lane 3);* CGG to CAG at codon 248 *(lane 4);* TGC to TAC at codon 242 *(lane 5);* GAA to CAA at codon 258 *(lane 6),* CGG to CAG at codon 248 *(lane 7);* ATG to ATA at codon 237 *(lane 8);* AGG to AGT at codon 249 *(lane 9),* and normal control *(lane 10)*

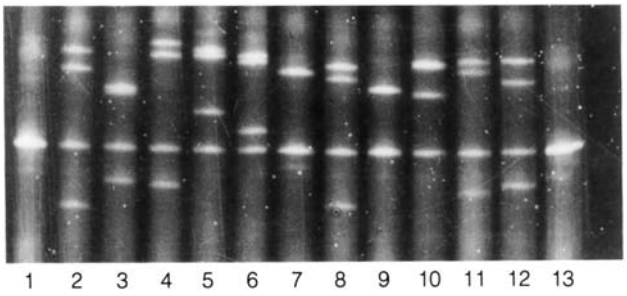

Fig. 4. DGGE analysis of randomly generated p53 mutations. Mutations were generated in a cloned p53 fragment using the method of Zhou et al. (1991). DGGE analysis of 11 mutations from the exon 7 region are shown *(lanes 2–12). Lanes 1* and *13* contain control samples. Each amplified mutant sample was mixed with amplified control DNA, denatured, and reannealled to form heteroduplexes. The presence of heteroduplexes increases the sensitivity of mutation detection by DGGE. The amplified samples were subjected to electrophoresis on a 35%–65% denaturing gradient gel for 6h

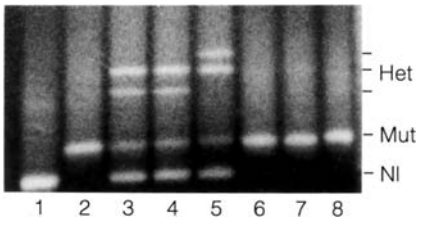

Fig. 5. DGGE analysis of amplified p53 mutations illustrating the value of heteroduplex formation in mutation identification. *Lanes 6, 7,* and *8* contain the PCR product from three cloned p53 mutants. *Lanes 3, 4,* and *5* contain the same three samples reannealled to the normal p53 sequence. *Lane 2* contains the same samples as *lanes 6* and *7* mixed, denatured, and reannealled together. *Lane 1* is a normal control. Note that the sample in *lanes 3* and *4* give the identical heteroduplex pattern indicating that they contain the same mutation (C to T substitution at the first nucleotide of codon 248). This is confirmed by the lack of heteroduplex bands when these samples are mixed and reannealled *(lane 2).*The sample in *lane 5* gives a different heteroduplex pattern indicating the presence of a different mutation (G to T at first nucleotide of codon 245). The mutant *(Mut),* normal *(Nl),* and heteroduplex *(Het)* bands are indicated

approach. Figure 3 shows DGGE analysis of nine DNA samples known to contain exon 7 p53 mutations based on sequence analysis. Mutants are recognized by the appearance of a shifted homoduplex band as well as by formation of heteroduplexes with the wild-type sequence. The heteroduplex DNA complex contains a mismatch, which destabilizes the molecule, resulting in bands that always run above homoduplexes in the gel. In addition to analysis of known mutations, DGGE analysis of randomly generated mutations in exons 5, 6, and 7 was performed. We were able to detect 37 mutations in 125 samples, consistent with the reported mutation frequency of the mutagenesis protocol (Zhou et al. 1991; see Fig. 4). Furthermore, DNA sequencing of a subset of the samples failed to identify mutations not detected by DGGE indicating that all the existing mutations were detected by the DGGE assay. In most cases, different mutations resulted in a different pattern of bands on a denaturing gradient gel. Occasionally, the homoduplex bands from two different mutations co-migrated. In such cases, heteroduplexes are particu-

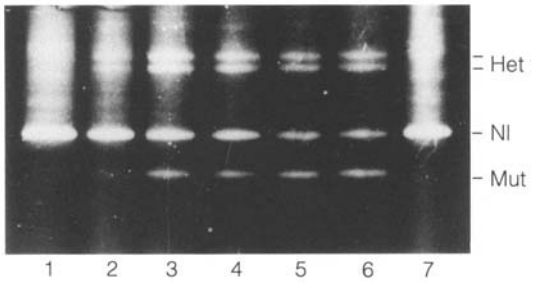

Fig. 6. Genomic DNA containing a p53 base substitution was mixed with normal genomic DNA and PCR amplified for 40 cycles. The amplified product was analyzed by DGGE. The proportion of mutant DNA to normal DNA is as follows: 5% mutant and 95% normal *(lane 1);* 12.5% mutant and 87.5% normal *(lane 2);* 25% mutant and 75% normal *(lane 3);* 37.5% mutant and 62.5% normal *(lane 4);* 50% mutant and 50% normal *(lanes 5 and 6);* 100% normal *(lane 7). Het* heteroduplex; *Nl* normal; *Mut* mutant

larly valuable for determining if two mutations are likely to be the same or different. The results shown in Fig. 5 illustrate this point. This figure shows the results of a mixing experiment performed to determine if three samples contain the same or different mutations. The formation of heteroduplexes in mixed samples indicates that the samples contain different mutations. The failure of heteroduplexes to form indicates that the two samples contain the identical sequence.

Detection of p53 mutations in tumor samples often requires the ability to detect mutations in tumor tissue contaminated by normal tissue. To determine the sensitivity of the DGGE assay for detecting mutant DNA in the presence of normal DNA, mutant DNA was mixed in varying ratios with normal DNA. The results shown in Fig. 6 demonstrate that 12.5% mutant DNA can be detected on a background of 87.5% normal DNA.

Samples that are heterozygous for a mutation (contain both a mutant and normal allele) are not efficiently sequenced using automated sequencing because the automated sequencer does not always recognize both the normal and mutant allele within a single sample. To overcome this problem, we tested the feasibility of cutting the mutant band from the denaturing gradient gel, eluting the DNA from the gel fragment, and using the eluted DNA as a template for PCR. This strategy results in isolation of the mutant allele from the normal allele. Figure 7A shows the purification of a mutant p53 allele from the normal allele using this approach and the subsequent automated sequencing of the separated alleles. Unambiguous sequencing results can be obtained using this approach (Fig. 7B).

Discussion

Techniques for identification of single-base changes include restriction enzyme analysis, allele-specific oligonucleotide hybridization, chemical cleavage, single-strand conformation polymorphism (SSCP) analysis, allele-specific PCR, heteroduplex analysis, and denaturing gradient gel electrophoresis. Many of these procedures are suitable for detecting a known change in a gene sequence, but are not suited for identifying many different changes within a gene sequence. For example, allele-specific PCR and allele-specific oligonucleotide hybridization require a separate oligonucleotide primer for each specific mutation assayed. Over 300 p53 gene mutations have been reported, making it difficult to screen for mutations in this gene with techniques that are aimed at identifying only a few

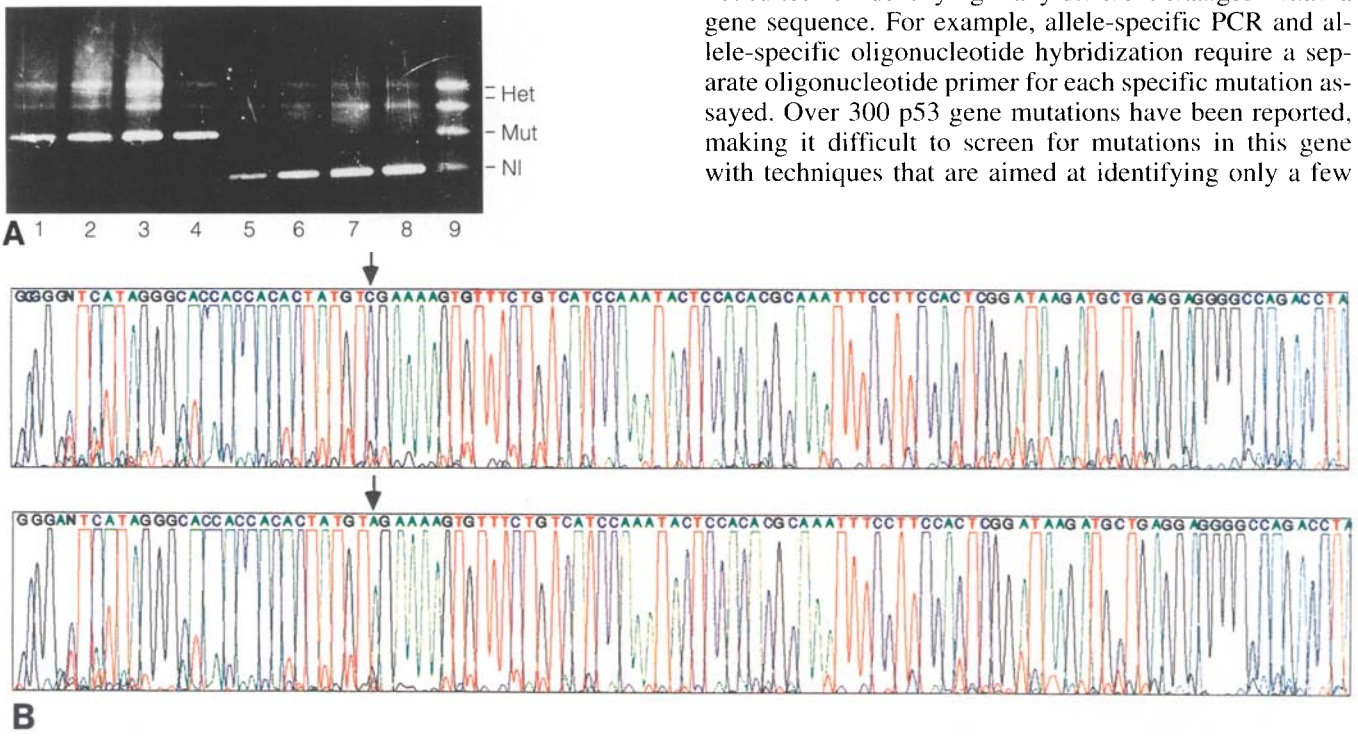

Fig. 7 A, B. Separation of a mutant exon 6 p53 allele from the normal allele in preparation for sequencing. The mutant and normal bands were excised from a denaturing gradient gel and the DNA was recovered by elution in TE8 buffer. The DNA was then used as template for reamplification with the original p53 exon 6 primers. The amplified product was then checked for purity by electrophoresis on a denaturing gradient gel (**A**) and sequenced using an ABI automated sequencer (**B**). Reamplified mutant allele *(lanes 1–4),* reamplified normal allele *(lanes 5–8),* original amplification product prior to allele separation *(lane 9).* The sequencing was performed using the reverse primer and shows a G to T (C to A in the sense direction) change at the second position of codon 213 *(arrows* in **B**)

specific mutations. DGGE and SSCP have been used to identify multiple different single-base substitutions in a variety of sequences (Sheffield et al. 1991, 1992; Hayashi 1992). Previous studies indicate that nearly all single-base substitutions can be detected by DGGE when a 40-bp G+C-rich sequence (GC-clamp) is attached by PCR to the DNA fragments to be analyzed (Myers et al. 1985b; Sheffield et al. 1989). In this study, we developed a specific GC-clamped DGGE assay for the p53 gene. This assay consists of PCR amplification of four DNA fragments containing five p53 exons (exons 5–9), followed by electrophoresis of the fragments on denaturing gradient gels. The region of the p53 gene surveyed for mutations by this assay contains the coding sequence for the highly evolutionarily conserved domains of the p53 protein. Over 98% of the reported p53 mutations lie within the four p53 fragments surveyed by this assay (Hollstein et al. 1991). Although this may partially be due to ascertainment bias, studies that examine other regions of the p53 gene demonstrate that mutations outside of exons 5–8 are rare (Nigro et al. 1989).

The major limitation of DGGE in detecting single-base substitutions is the inability of this approach to detect some mutations that lie within the highest melting domain of a fragment. This limitation is overcome in this assay because the analyzed sequence of each fragment falls within a single domain adjacent to a high GC-clamp melting domain. Thus on theoretical grounds, this assay should detect all single-base substitutions in the sequence lying between the primers of each PCR product. The experimental data of this study confirmed the high sensitivity of this assay. Of the multiple known p53 mutations analyzed in this study, all were detected by DGGE. In addition, 37 randomly generated p53 mutations were detected by this DGGE assay, further demonstrating that this p53 mutation assay can detect a wide variety of mutations. Furthermore, an earlier version of this assay has been used to detect novel p53 mutations from tumor tissue (Cogen et al. 1992). A p53 assay using a modification of DGGE known as constant denaturant gel electrophoresis (CDGE) has been reported (Børresen et al. 1991). However, the CDGE assay screens a smaller portion of the p53 gene for mutations, and requires the use of two different gel conditions for some of the fragments analyzed.

The DGGE approach described in the present study can be used as an efficient screening assay for mutations within the p53 gene. Once a change is indicated by DGGE, the specific fragment containing the mutation can be sequenced. This eliminates the need to sequence large regions of the gene; only the fragment known to contain a mutation as determined by DGGE need be sequenced. Furthermore, DGGE can be used in many cases to separate the mutant from the normal allele thus facilitating sequencing of the mutant allele. In addition, different mutations give different patterns of bands by DGGE analysis. Some of the more common mutations can be readily recognized and confirmed without sequencing by mixing a known mutation with the sample suspected to have the same mutation. Many tumor samples that potentially harbor p53 mutations are highly contaminated by normal tissue. This decreases the sensitivity of detection of p53 mutations by direct DNA sequencing. However, the p53 GC-clamped DGGE assay can be used to detect mutations with a high degree of sensitivity in tumor samples that are contaminated with normal tissue. This assay should prove useful in clinical studies seeking to establish the clinical significance of specific p53 mutations, as well as for screening of patients suspected to be at risk for carrying germline p53 mutations.

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