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Detection of p53 Gene Mutation in Cancer Tissues by Nonradioactive Direct Sequencing

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A simple procedure for direct sequencing of double-stranded PCR products by the dideoxy-termination method has been developed using biotinylated sequencing primers. Sequences of the p53 gene have been obtained from DNA extracted from frozen and formalin-fixed paraffin-embedded cancer tissues. Detection of sequencing ladders was done with chemiluminescent or colorimetric techniques. Both are highly sensitive, but colorimetric detection is less prone to diffusion artifacts, which are common in G-rich regions. Use of this nonradioactive PCR-sequencing protocol allowed rapid and simple determination of p53 gene alterations in human tumors.

PCR amplification of human DNA is a very specific and sensitive approach to the study of genomic alterations in cancers. In most cases, definitive analysis of these alterations requires DNA sequencing. Cloning and direct sequencing of asymmetrically amplified PCR products are commonly used techniques.[1] Cloning is time consuming and carries the risk of misidentification of mutations because of PCR-induced errors. Methods used to generate single-stranded templates from PCR products, such as asymmetric amplification,[2,3] affinity capture by immobilization on magnetic beads,[4] or λ exonuclease digestion,[5] require complex manipulations to produce a template of sufficient quality and quantity. None of these techniques reliably produced products that could be sequenced. In contrast, double-stranded sequencing of PCR-amplified DNA can resolve these technical problems.[6] Furthermore, the same PCR products can be used prior to sequencing for screening genetic alterations by single-strand conformation polymorphism (SSCP).[7]

For the p53 gene, single-stranded sequencing has an additional difficulty, because of its relatively high content of GC-rich regions, producing frequent stops at sites of secondary structures. The high temperature of the double-stranded sequencing reaction with *Taq* polymerase could avoid such artifacts.

The main difficulties for direct sequencing are the presence of nonspecific amplification products, excess primers, and deoxyribonucleotide triphosphates (dNTPs) carried over from the amplification step, which necessitate purification of the PCR product before sequencing. The concentration and purity of template seem to be the most important factors in determining the efficiency and reliability of direct double-stranded sequencing.[1]

Direct sequencing of PCR-amplified DNA has been the focus of many recent studies (4–6, 8–15). It gives a maximum amount of information about the PCR template while avoiding complex procedures. Radioactive detection, used in most protocols, is expensive, necessitates complex safety measures, and is slow because of the extensive time required for autoradiography. Chemiluminescent[8, 16–19] and color reactions are alternative detection methods to radioisotopes that avoid such difficulties.

We have developed a protocol for nonradioactive analysis of PCR-amplified p53 gene with direct sequencing of double-stranded product. Using biotin-labeled oligonucleotides as sequencing primers, we are able to fix a biotin–streptavidin–alkaline phosphatase chain to sequenced DNA fragments. Alkaline phosphatase allows a further direct chemiluminescent and/or color detection. This procedure is simple, rapid, and reliable and avoids technical problems linked to isotopic detection.

MATERIALS AND METHODS

Double-stranded DNA Amplification

DNA was obtained from frozen and formalin-fixed paraffin-embedded gastric cancer biopsies. DNA was extracted from frozen tissues using a Elu-Quik DNA Purification Kit (Schleicher & Schuell, Keene, NH) according to the manufacturer's instructions. The complete process was carried out in 1 hr. Tumoral regions from paraffin-embedded tissues

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were removed by scraping with a sterile scalpel and boiled at 100°C for 10 min in TE buffer (10 mM Tris-HCl, 1 mM EDTA at pH 8).

Exons 5–8 of the p53 gene were amplified separately. Each PCR was performed using 30 μl final reaction volumes containing 3 μl of extracted DNA, 0.5 μM of each primer (Fig. 1), 1.5 μl (250 μM) of deoxyribonucleotide triphosphate (dNTP) mixture, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.75 mM $MgCl_2$, and 0.8 units of *Taq* DNA polymerase (Boehringer Mannheim). Mixtures were subjected to 35 cycles of amplification in a Perkin-Elmer DNA Thermal Cycler model 9600, each consisting of 30-sec denaturation at 94°C, 45-sec annealing at 55°C (exons 5,7, and 8) or 60°C (exon 6) and 45-sec polymerization at 73°C.

When necessary, DNAs from specimens that showed a change in the migration pattern were reamplified using 2 μl of PCR as template in the same conditions in a final volume of 60 μl.

The PCR product was loaded onto a 2% low-melting agarose gel (FMC NuSieve, Rockland, ME) containing ethidium bromide and electrophoresed at 2 V/cm for 30 min. Under UV light, appropriate bands were excised using a sterile scalpel and purified using Magic PCR Preps (Promega, Madison, WI) according to the recommendations of the supplier.

Direct Sequencing Analysis

One-third of the purified product was used for cycling sequencing reaction following the AmpliTaq Cycle Sequencing Kit (Perkin Elmer Cetus, Norwalk, CT) protocol. The reaction mixture (20 μl) consisted of 1.6 pmole of sequencing primer biotin-labeled at the 5′ end, 100 mM Tris-HCl (pH 8.8), 100 mM KCl, 5 mM $MgCl_2$, and 2 units of AmpliTaq DNA polymerase. This mixture was dispensed into four 4-μl aliquots and mixed with 4 μl of the respective dideoxyribonucleotide triphosphate termination mixtures. The reactions were heated at 95°C for 15 sec and 20 cycles each of 10 sec at 95°C and either 25 sec at 55°C (exons 5,7, and 8) or 60°C (exon 6). The reactions were then stopped at 4°C by addition of 4 μl of formamide solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.02% xylene cyanol FF). Sequencing reaction products were stored at −20°C. Prior to electrophoresis, products were heated at 95°C for 5 min. Then, 3.5 μl of each reaction was loaded onto a 6% Long Ranger gel (AT Biochem, Malvern, PA) and electrophoresed at 40 W for 2 hr in a Bio-Rad Sequi-Gen apparatus (Hercules, CA).

Exon 5	5′-TTCCTCTTCCTACAGTAC-3′ *
	5′-ACCTCTCTGCTGTCCCGA-3′
Exon 6	5′-GGGCTGGTTGCCCAGGGT-3′
	5′-ACTCCAGACCAAACGTTGA-3′ *
Exon 7	5′-GTGTTATCTCCTAGGTTG-3′
	5′-CAGTCCTCGGTGAACGGT-3′ *
Exon 8	5′-CCTATCCTGAGTAGTGGTAA-3′
	5′-TGTTCTTCGCCACCTCCT-3′
	5′-TCCATTCGTTCGTCCTGT-3′ *

FIGURE 1 Primers used for PCR and sequencing. Asterisks (*) indicate biotinylated sequencing primers.

After electrophoresis, the gel was left attached to one glass plate. DNA was capillary transferred by placing a wet (0.5× TBE) nylon membrane (Immobilon-S, Millipore, Bedford, MA; or Nytran NY-13-N, Schleicher & Schuell) on the gel surface. Two sheets of dry 3MM Whatman filter paper, a glass plate, and a moderate weight were added on top. Transfer was completed in 20 min. Under these conditions, approximately one-third of the DNA products were transferred to the membrane. The membrane was then dried at 50°C for 15 min, and DNA was cross-linked by UV irradiation at 302 nm for 5–10 min.

Detection

Chemiluminescent detection was performed using the PHototope Detection Kit (New England Biolabs) according to the manufacturer's instructions. The protocol was carried out in large cylinders on horizontal rollers at room temperature. The membrane was first incubated with blocking buffer (5% SDS, 17 mM Na_2HPO_4, 8 mM NaH_2PO_4 at pH 7.2), followed by an incubation with streptavidin (1 μg/ml in blocking buffer) and washed twice with 1:10 diluted blocking buffer. The membrane was incubated with biotinylated alkaline phosphatase solution (0.5 μg/ml in blocking buffer) and washed twice with wash solution (10 mM Tris-HCl, 10 mM NaCl, 1 mM $MgCl_2$ at pH 9.5). All incubation and washing steps were 5 min long. The membrane was placed in a plastic bag with the substrate solution (Lumigen-PPD) in the dark. After a waiting period of 30 min, the membrane was exposed to X-ray film (X-OMAT, Kodak) for 45 min and developed.

Alternatively, the membrane was subjected to a color reaction before or even after the addition of chemiluminescent substrate (Lumigen-PPD). The membrane was equilibrated for 2 min with 50 ml of alkaline buffer (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$ at pH 9.5) and incubated in the dark with freshly prepared alkaline phosphatase substrate solution (0.033% of NBT and 0.015% of X-phosphate in alkaline buffer). When the desired bands were prominent enough, generally after 1 or 2 hr, the reaction was stopped by washing the membrane in buffer 2 (10 mM Tris-Hcl, 1 mM EDTA at pH 8). 89

RESULTS AND DISCUSSION

We have developed a protocol for nonradioactive analysis with direct sequencing of PCR-amplified exons 5–8 of the p53 gene. The method can be applied to DNAs extracted from frozen or formalin-fixed paraffin-embedded tissues. The complete procedure is carried out in 24–36 hr (Fig. 2). In a first approach, screening for genetic alterations in exons 5–8 of the p53 gene was done by nonradioactive PCR–SSCP analysis of DNA extracted from cancer tissues.[4] Subsequently, DNA showing an altered band pattern was gel purified and sequenced. Nonradioactive direct sequencing of double-stranded DNA was performed using biotinylated primers and *Taq* DNA polymerase for extension. (Commercial biotin-labeled oligonucleotides are stable for years, making it unnecessary to label them before each reaction). The reaction products were separated by polyacrylamide gel electrophoresis, transferred directly to a nylon membrane, and fixed by UV light. Detection of sequencing ladders was done by chemiluminescent and/or color reactions after binding streptavidin–alkaline phosphatase to the biotinylated DNA fragments.

To find a reproducible method for direct sequencing of the PCR-amplified

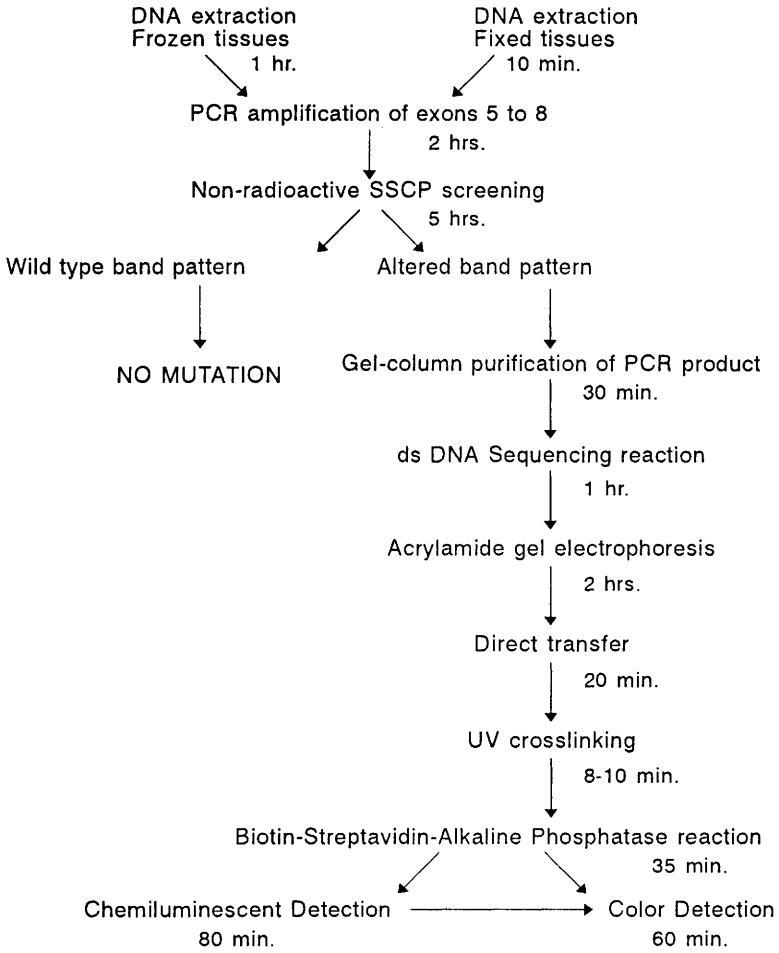

FIGURE 2 Protocol of direct nonradioactive analysis of p53 gene alterations.

p53 gene we have tested many published techniques[2–5] to obtain a good quality single-stranded template. In our experience, single-stranded DNA direct sequencing with Sequenase is not a reproducible approach to study p53 gene alterations because of relatively high content of GC-rich regions within the gene. Double-stranded sequencing with *Taq* polymerase helps to resolve this problem and thus provides a reproducible method to direct sequencing of 165- to 288-bp PCR products (amplified exons 5–8 of the p53 gene).

Because the main difficulties with direct double-strand sequencing are nonspecific amplification products and exess of primers and dNTPs, purification of PCR product prior to the sequencing reaction was essential. Unlike other investigators,[9] we were not able to obtain a good quality of sequencing reactions without purification of the amplified product. Good results have been obtained by phenol extraction and ethanol precipitation of PCR product, but both are time consuming. For PCR-amplified products >160 bp, Magic PCR Preps (Promega) purification kit provides a good quality of final product in 20 min. In cases of smaller products, we used a classic phenol extraction after band isolation in a low-melting agarose gel with similar results.

For sequence analysis of exon 8, a biotinylated sequencing primer was internal to amplification primers (nested primer). In other exons, the sequencing primer coincided with one of the amplification primers. There were no differences in results in both situations.

We have tested different membranes and transfer methods to find a better quality of membrane transfer. Better results were obtained by direct transfer for 20 min on Immobilin (Millipore) or Nytran NY-13-N (Schleicher & Schuell) nylon membranes. The first provided a sharp contrast in chemiluminescent detection, but color reaction resulted in fuzzy bands. In contrast, the Nytran membrane gave excellent results with both detection techniques. Direct transfer decreased band diffusion relative to other methods and avoided the use of special vacuum apparatus and long transfer techniques.

In our experience, UV cross-linking of the DNA onto the membrane is an important step in detection of sequencing reaction. Very short exposure times gave a general weak fixation, whereas extensive times produced a loss of signal intensity more pronounced for small fragments. It is important, therefore, to optimize the cross-linking time for each UV lamp.

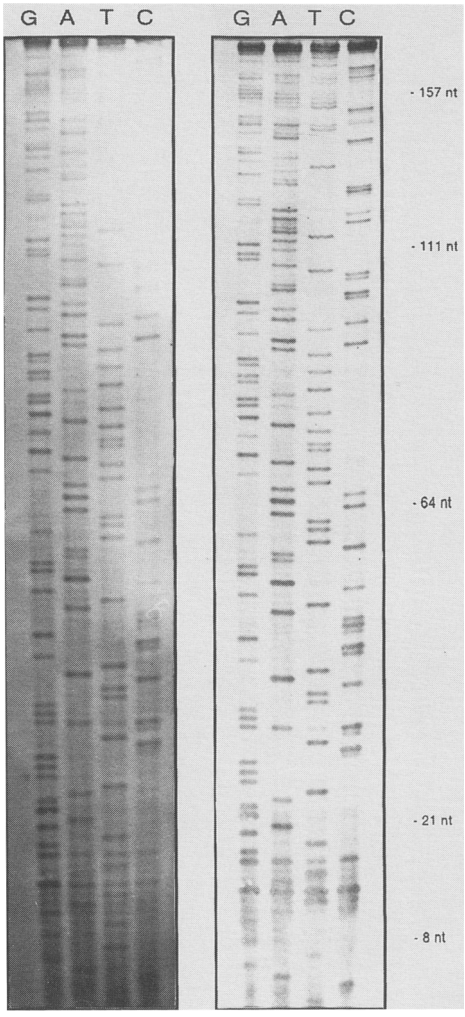

FIGURE 3 Comparison of sequencing by chemiluminescent or colorimetric detection. Chemiluminescent (*left*) and color (*right*) detections were performed successively on the same nylon membrane to analyze the sequence of exon 7 of the p53 gene in an archival formalin-fixed paraffin-embedded gastric cancer tissue. No mutation was found in this exon.

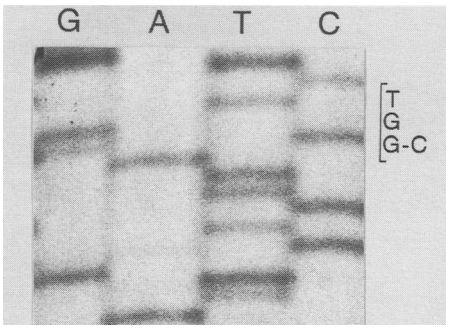

FIGURE 4 Example of a p53 gene mutation in an archival gastric carcinoma. A transversion from GGT (glycine) to CGT (arginine) at codon 187 (exon 6) was visualized by chemiluminescent detection.

Chemiluminescent detection increases the efficiency of sequencing over isotopic methods, because AMPPD is a stable reagent and exposure times are considerably shorter. Resolution and sensitivity are similar to radioactive detection.

Color detection also provides excellent sensitivity and avoids diffusion artifacts, especially important in G-rich regions, with a sharp final band image. Another advantage of color detection is a uniform quality of the resolved bands (Fig. 3). It is not necessary to have a photographic laboratory, and detection time is similar to cheiluminescent reaction.

Using this procedure, p53 mutations have been detected easily from either frozen or fixed cancer tissues (Fig. 4) with relatively high sensitivity. This sensitivity in detection of mutant DNA is determined by the SSCP screening, where minimal proportion of detectable mutated p53 alleles is ~15%.[7]

In this protocol the complete study of a tumor sample for p53 mutations can be carried out in 3 days without the use of radioactive methods. Its simplicity and reliability in the analysis of PCR-amplified DNA from either frozen or paraffin-embedded tissues shows its usefulness in the determination of genetic mutations in cancer series studies.

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