Radiolabeled Polymerase Chain Reaction Assay for Detection of ras Oncogene Point Mutations in Tumors

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The human ras gene plays a fundamental role in the transduction of extracellular signals to the nucleus, thereby regulating cell growth and differentiation. Point mutations in the ras gene convert it into a transforming oncogene that has been found in many solid and hematologic malignancies. We describe a rapid and sensitive assay based on a radiolabeled polymerase chain reaction followed by restriction enzyme digestion that we have adapted for differentiating between the wild-type and mutant ras genes. This assay should prove useful in the analysis of ras gene point mutations in clinical tumor specimens in which ras oncogene activation is an early event in carcinogenesis.

Indexing Terms: hybridization assays/restriction enzymes/cancer/single-strand conformation polymorphism

The human ras gene family includes the Ha-, Ki-, and N-ras genes, all of which encode homologous GTP binding proteins that play a role in signal transduction. Point mutations in the ras gene, particularly in codons 12, 13, or 61, convert the gene into a transforming oncogene, and have been described in a variety of tumors (1, 2). Numerous techniques have been used for the detection of ras point mutations in tumor specimens. The biological assay based on DNA transfection into NIH/3T3 cells and subsequent identification of cells that either form morphologically transformed foci or induce tumors in nude mice is too laborious to be applicable for large-scale clinical screening. Bos et al. (3, 4) developed a dot-blot procedure for the identification of ras gene point mutations by hybridization to specific oligonucleotide probes. Jiang et al. (5, 6) devised a nonradioactive method, using polymerase chain reaction (PCR)-amplified DNA with mismatched Ki-ras primers followed by restriction enzyme digestion to distinguish between mutant and wild-type alleles. Kumar and Barbacid (7) described an assay involving PCR-amplified Ki-ras DNA fragments and a liquid hybridization technique with labeled oligonucleotide probes. Others have used a PCR/Southern-based assay (8).

By using radioactive dNTPs and mismatched Ki-ras primers, we have adapted a PCR-based assay to distinguish between wild-type and mutant DNA templates after restriction enzyme digestion. We have compared this assay with other techniques and show that it is a rapid and sensitive method for detecting ras gene point mutations in a heterogeneous cell population.

Materials and Methods

Colon carcinoma cell lines and genomic DNA preparation. Fourteen colon carcinoma cell lines (COLO320HSR, DiFi, Caco-2, HCT116, COLO205, T84, LoVo, DLD-1, COLO201, SK-CO1, LS-174T, SW1417, SW480, and HT29) were obtained from American Type Culture Collection (Rockville, MD). High-molecular-mass DNA was prepared from these cell lines (9).

PCR amplification. One microgram of genomic DNA was amplified in a 100-µL reaction volume containing 10 mmol/L Tris-HCl (pH 9.0), 50 mmol/KCl, 1.5 mmol/L MgCl2, 0.1 g/L gelatin, 1.25 mmol/L dNTPs, 1 µmol/L PCR primers, and 2.5 U of Taq polymerase (Promega, Madison, WI). We used 5'-ACTGAATATAACCT-TGTGGTAGTTGGACCT-3' (KRAS5') as the 5' primer and 5'-TCAAGGATGGTCTGGACC-3' (KRAS3') as the 3' primer (6). A PCR cycle consisted of 1 min of denaturation at 94°C, 2 min of annealing at 55°C, and 3 min of elongation at 72°C. Samples were subjected to 35 cycles of amplification.

Restriction enzyme analysis. Aliquots (10 µL) of PCR-amplified samples were digested with the restriction enzyme BstNI in a total volume of 20 µL under conditions recommended by the supplier (New England Biolabs; Beverly, MA). Digestion mixtures were incubated at 60°C for 2–3 h. DNA was electrophoresed through an 8% native acrylamide gel for 4–5 h. Gels were stained with ethidium bromide and photographed on an ultraviolet light transilluminator.

Liquid hybridization and probe shift assay. This assay was based primarily on the previously described method (7). Five microliters of the PCR sample was digested with 2 µL (16 U) of BstNI at 60°C for 12–16 h to detect the Ki-ras codon-12 mutation. For detection of the Ki-ras codon-13 mutation, 10 µL of PCR product was digested with 2 µL (8 U) of HphI (New England Biolabs) at 37°C for 14–16 h. The digest was mixed with 5 x 10^6 dpm of the Ki-ras first exon probe (5'-CGAATATGATTGCACAAATAG-3') (KRASY) (Fig. 1) in a 30-µL reaction volume containing 0.75 mol/L NaCl. The probe was end-
labeled with [γ-32P]dATP (6000 Cimol/L) (New England Nuclear, Boston, MA) and T4 polynucleotide kinase (Pharmacia LKB Biotechnology, Piscataway, NJ). Reactions, overlaid with mineral oil, were denatured at 97°C for 10 min and rapidly cooled to the annealing temperature of 55°C. Hybridization was carried out at 55°C for 2 h and terminated by cooling on ice. Separation was carried out on an 8% native acrylamide gel by electrophoresis. After electrophoresis, the gel was placed in a plastic wrap and exposed to Amersham (Arlington Heights, IL) Hyperfilm™ at room temperature for 4–6 h.

Southern hybridization with specific oligonucleotide probes. Southern hybridization for detecting the Ki-ras valline mutation at codon 12 was performed according to the previously described method (8, 10). Briefly, 50 µL of each PCR sample was run on a 2% agarose gel, and, after incubation in 16 mmol/L HCl for 30 min, the gel was transferred to Immobilon N (Millipore, Bedford, MA) in 0.4 mol/L NaOH. The oligonucleotide used for hybridization was 5'-GGAGCTGTTGGCGTAGGCAA-3' for the valine codon-12-mutant Ki-ras. The oligonucleotide was labeled with 32P and T4 polynucleotide kinase to a specific activity of ~108 dpm/µg. Hybridization was performed at 52°C for 6 h. After hybridization, the membranes were rinsed in 3x SSC (450 mmol/L sodium chloride, 18 mmol/L sodium citrate, and 1 mmol/L Tris-HCl, pH 7.2), 1 g/L sodium dodecyl sulfate at room temperature for 5 min, and then washed at 56°C in 3x SSC, 1 g/L sodium dodecyl sulfate for 30 min. The filters were then exposed to film at ~70°C for 2–6 h.

Radioactive PCR. Radioactive PCR was carried out in a 100-µL volume with the following modifications: The concentration of cold dCTP was reduced to 0.25 mmol/L, and 0.5 µL of [α-32P]dCTP (3000 Cimol/L) (New England Nuclear) was added to the reactions. After amplification, 5 µL of each PCR sample was digested with 2 µL (16 U) of BstNI in a 20-µL volume at 60°C for 14–16 h. The digests were run on an 8% native acrylamide gel for 4–5 h. The gel was wrapped in plastic wrap and exposed to film at room temperature for 2–6 h.

Single-strand conformation polymorphism (SSCP) analysis. Ki-ras codon-12 mutation was detected with a modified version of the SSCP method (10, 11). Following radioactive PCR as described above, 2 µL of the PCR product was mixed with 8 µL of loading buffer (950 mOL formamide, 20 mmol/L EDTA, 0.5 g/L xylene cyanol, 0.5 g/L bromphenol blue) and incubated at 95°C for 5 min. We loaded 2 µL of each sample on a 6% acrylamide gel containing 100 mOL/glycerol. The gel was run at 40 W constant power for 3–4 h at 4°C with 1x TBE (89 mmol/L Tris-borate, 2 mmol/L EDTA, pH 8.3) as running buffer. The gel was dried and exposed to film at ~70°C for 14 h.

Results

Detection of Ki-ras codon-12 and -13 mutations in colon carcinoma cell lines. Genomic DNA prepared from 14 colon carcinoma cell lines was amplified with the primers KRAS5' and KRAS3', generating a DNA fragment of 157 nucleotides. The KRAS' primer is 30 nucleotides long and incorporates a cytosine residue at the first position of codon 11 and terminates adjacent to codon 12 (Fig. 1). A single nucleotide substitution is also incorporated into the KRAS3' primer as a positive control for BstNI cleavage. The Ki-ras codon-12 mutation was detected with BstNI digestion and liquid hybridization in the SW480, SK-CO1, and LS-174T cell lines (Fig. 2). Upon incubation with BstNI, fragments encoding wild-type codon-12 sequences were cleaved twice, resulting in the largest band of 114 nucleotides. Fragments containing mutations at either the first or second positions of codon 12 were cleaved only once, resulting in a longer band of 143 nucleotides (Fig. 1). In the SW480 cell line, only the mutant band was observed and this is consistent with the previously reported results.

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Fig. 1. Fragment sizes created by restriction enzyme digestion in Ki-ras codons 12 and 13.

The PCR fragment with wild-type Ki-ras codon 12 is digested twice by BstNI, once at the BstNI site that overlaps codon 12 and once at an internal control site at the 3' end, creating three bands of 114, 29, and 14 bp. A mutation in either of the first two bases of Ki-ras codon 12 destroys the BstNI site, and therefore the DNA is digested only once at the internal control site, creating two bands of 143 and 14 bp. Digestion with HphI detects an aspartic acid mutation at Ki-ras codon 13, because the substitution of A for G at the second position of codon 13 creates a HphI restriction site. A point mutation is digested, creating 114 and 43 bp bands, while the wild-type sequence remains undigested. The PCR sample is digested with BstNI or HphI, followed by liquid hybridization with a radioactively labeled oligonucleotide probe KRAS and subsequent gel analysis, as described in Materials and Methods.
that the SW480 cell lines is homozygous for the codon-12 valine allele (12). In the SK-C01 and LS-174T cell lines, both the mutant and the wild-type bands were observed, indicating the presence of both mutant and wild-type alleles (Fig. 2).

The Ki-ras codon-13 aspartic acid mutation was screened with the same PCR products from 11 colon carcinoma cell lines. The PCR fragment was digested with the restriction enzyme HphI. Transition to an adenosine residue at the second position of codon 13 results in the substitution of glycine to aspartic acid and creates an HphI site. Wild-type DNA fragments remained undigested, while codon-13 aspartic acid mutants were cleaved to a 114-nucleotide band (Fig. 1). The mutant band was observed in the HCT116, T84, and LoVo cell lines (Fig. 3). These cell lines also have the wild-type band; no cell line tested was homozygous for the aspartic acid allele of the Ki-ras codon 13.

Restriction enzyme analysis. Genomic DNA from the SW480 colon carcinoma cell line, which is homozygous for the Ki-ras codon-12 valine mutation, was serially diluted with genomic DNA prepared from the HT29 colon carcinoma cell line, which has a wild-type Ki-ras gene. These mixtures were amplified by PCR. PCR-amplified samples digested with BstNI were run on an 8% native acrylamide gel. As expected, only the 143-nucleotide mutant band and the 114-nucleotide wild-type band were visible in the 1:0 and the 0:1 dilutions, respectively. The 143-nucleotide mutant band was still detectable in the 1:32 dilution (data not shown). Previously, the sensitivity for K-ras codon-12 mutation with the same method was reported as 1:16 dilution (5). While this method is simple and nonradioactive, it is not sensitive enough to detect a ras mutation in clinical samples with a vast excess of normal cells.

Liquid hybridization assay. We compared four different methods for sensitivity and specificity of detection of the Ki-ras codon-12 mutation. All methods were performed at least five times and conditions optimized as reported in previous studies (5–8). PCR fragments were digested with BstNI and the digest was mixed with the K-ras first exon probe (KRASY) (Fig. 1) for liquid hybridization (7). Hybridization of the radiolabeled probe to the target-amplified DNA results in the formation of hemiduplexes, which can be separated from the free probe by electrophoresis in the native acrylamide gel. The 143-nucleotide mutant band was still detectable in the 1:10³–10⁴ dilution (Fig. 4). A potential problem with this assay is the generation of false-positive results due to incomplete digestion of the sample with BstNI. To try to eliminate this problem, we used only 5 µL of the 100-µL PCR product and digested it for 14–16 h with

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**Fig. 2.** Screening of colon carcinoma cell lines for mutant Ki-ras codon 12.
Genomic DNA (1 µg) from 14 colon carcinoma cell lines and two normal human lymphocytes was amplified with KRAS5' and KRAS3' primers and analyzed for codon-12 mutations with BstNI digestion followed by liquid hybridization. Lanes marked normal and uncut refer to BstNI-digested and undigested amplified normal human lymphocyte DNA, respectively. Numbers on the left denote the sizes of DNA bands. On the right, the positions of the uncut and BstNI-digested mutant and wild-type Ki-ras codon-12 fragments are labeled. In the SW480 cell line, only the mutant band is seen. In the SK-C01 and LS-174T cell lines, both the mutant and wild-type bands are present.

**Fig. 3.** Screening of colon carcinoma cell lines for a mutant aspartic acid Ki-ras codon 13.
Genomic DNA (1 µg) from 11 colon carcinoma cell lines was amplified with KRAS5' and KRAS3' primers and the aspartic acid mutation of Ki-ras codon 13 was detected with HphI digestion followed by liquid hybridization. The lane marked uncut refers to undigested amplified HT29 colon cancer cell line DNA. Numbers on the left denote the sizes of DNA bands. The positions of the uncut and HphI-digested mutant and wild-type Ki-ras codon 13 fragments are labeled. The mutant band is seen in the HCT116, T84, and LoVo cell lines.

**Fig. 4.** Detection of Ki-ras codon 12 mutation.
SW480 DNA was mixed with HT29 DNA in 10-fold serial dilutions. Mixtures were amplified and products were then digested with BstNI and hybridized with KRASY probe in a liquid condition. Ratios of SW480 to HT29 DNA are given above the sample lanes. The migration of uncut, mutant, and wild-type bands is indicated.
BetNI, as our initial results showed that even 8 h of digestion of the 5-μL PCR product with BetNI generated incompletely digested bands. Although 14–16 h of digestion improved sensitivity, there were persistent problems with background bands (Fig. 4).

**PCR and Southern hybridization.** In this method, extremely stringent hybridization and washing conditions are required to ensure that the mutant oligonucleotide (differing at a single base from the wild-type sequence) hybridizes only to mutant molecules, resulting in the appropriate signal-to-noise ratio. Even slightly nonideal conditions led to false-positive results, and too stringent conditions decreased the sensitivity. Although this method has been successfully used to identify mutant ras molecules in stool samples from patients with large colonic tumors, its sensitivity remains undocumented (8). We used the sequence-specific oligonucleotide with a high specific activity (10^9 dpm/μg) and chose the stringent hybridization and washing conditions described above. In our optimized conditions for this assay, the mutant signal was still visible in the 10^2–10^3 dilution range (Fig. 5).

**Radiolabeled PCR.** Amplified PCR fragments were labeled by incorporating [α-32P]dCTP, digested with BetNI, and separated by electrophoresis on an 8% acrylamide gel. Our results indicate that optimal conditions require 5 μL of the 100-μL PCR product digested with BetNI. Using too much product generated false-positive bands and too little product decreased sensitivity. In this manner we could detect the 143-nucleotide mutant band in the 1:10^5–10^6 dilution (Fig. 6). This method is easier compared with the other two radioactive methods (liquid hybridization and Southern hybridization) and has greater sensitivity. Furthermore, each band was evident without background signals and results could be obtained within 24 h.

**SSCP analysis.** To simplify detection of Ki-ras mutation, we also ascertained the sensitivity of the SSCP analysis. 32P-labeled radioactive PCR product was prepared with the same PCR amplification conditions as described previously. As shown in Fig. 7, the band with different migration was observed only in the 1:10 dilution. Generally, if the sample contains >10% of mutant molecule, it is possible to detect the mutation by SSCP analysis (11, 12). SSCP is limited in detection of the ras mutation when wild-type ras molecules constitute >90% of the clinical sample.

**Discussion**

Many molecular biological approaches have been used to analyze point mutations in the ras gene and applied to
the early diagnosis of tumors or to differentiating benign from malignant conditions (18). Using radioactive dNTPs and mismatched Ki-ras oligonucleotides, we have adapted a PCR-based technique to differentiate between wild-type and mutant Ki-ras DNA templates.

The PCR/Southern assay involves amplification of Ki-ras codons 12 or 13 and detection of mutant molecules by hybridization with labeled oligonucleotide probes. The specificity of the method depends on conditions that ensure that the mutant oligonucleotide provides a detectable signal only by hybridizing to mutant molecules. Stringent hybridization and washing conditions are required for the appropriate signal-to-noise ratio. Radiolabeled PCR followed by restriction enzyme digestion proved to be faster and more sensitive than the PCR/Southern assay.

A liquid hybridization approach involving PCR amplification with mismatched oligonucleotides, diagnostic restriction enzyme digestion, and detection with a radiolabeled probe was extensively examined. The radiolabeled PCR assay we describe is an adaptation of this technique. Although the liquid hybridization assay is as quick and sensitive as the radiolabeled PCR method, its applicability is limited by higher background noise, making interpretation difficult. Furthermore, hybridization and washing steps are obviated in the radiolabeled PCR method. An SSCP-based approach has low sensitivity when compared with all other techniques tested, and thus its application to clinical material is limited.

We conclude that the radiolabeled PCR method followed by diagnostic restriction enzyme digestion is a rapid and sensitive means of detecting ras gene point mutations (Table 1). This method has the advantages of no hybridization steps and a high signal-to-noise ratio. Conditions for all methods were meticulously optimized as reported in the literature. The radiolabeled PCR method is especially useful for analyzing clinical samples in which mutant ras alleles represent a small percentage of available ras molecules. Such clinical situations are important in the early detection of cancer and in the differentiation of benign from malignant disease. Applications in pancreatic cancer and colon cancer are under investigation.

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References

Table 1. Comparison of radioactive methods.

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