Rapid, Nonradioactive Screening for Activating *ras* Oncogene Mutations Using PCR-primer Introduced Restriction Analysis (PCR-PIRA)

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None of the mutations in codons 12, 13, or 61 of the *K-ras*, *N-ras*, and *H-ras* genes, which convert them into active oncogenes,⁽¹⁾ creates or destroys a new restriction site for any known enzyme. A method based on the introduction of an artificial restriction site into a PCR product by the use of a primer containing a single-base mismatch at the 3' end has been used to detect a point mutation in codon 12 of *K-ras*.⁽²⁾ We have expanded this strategy to develop a simple screening system for activating *ras* mutations, termed PCR-primer introduced restriction analysis (PCR-PIRA).

METHODS

DNA was isolated from cells containing known *ras* mutations, obtained from the American Type Culture Collection [HT 1080 (heterozygous *N-ras* codon 61 CAA→AAA), SW 480 (homozygous *K-ras* codon 12 GGT→GTT), MOLT-4 (heterozygous *N-ras* codon 12 GGT→TGT)],⁽³⁾ from lymphoid tissue from a patient with γ -immunoglobulin heavy-chain disease (HCD), and from normal controls. Genomic DNA, 0.125 μ g, was PCR-amplified (94°C x 45 sec, 55°C x 45 sec x 30 cycles)⁽⁴⁾ across *K*- and *N-ras* codons 12, 13, and 61 using previously described primers.⁽⁵⁾ Secondary PCR was then performed on 1 μ l of a 1:1000 dilution of the initial PCR product, using the same 3' primer as well as a nested 5' primer, which contained a one- or two-base mismatch with the original PCR product and introduced a new restriction site into the second PCR product, such that a mutation in the codon being tested led to the loss of the new site (Table 1). In the secondary PCR, annealing was performed at 50°C to allow uniform primer binding despite the mismatches. Secondary PCR products were digested with the appropriate endonucleases and electrophoresed on 12% acrylamide (Fig. 1) or 2.5% agarose (Figs. 2 and 3) gels containing ethidium bromide. This protocol yielded complete digestion of the normal sequence, partial digestion with one normal and one mutated allele, or no digestion in the absence of a normal allele.

RESULTS

Each two-step PCR yielded a product of

the expected length which was cut by the appropriate restriction enzyme in the absence of a mutation, demonstrating the validity of the method (Figs. 1-3). The primer pairs used in the secondary PCR could also be used to amplify genomic DNA directly, but the two-step method produced higher, more specific yields.

A typical heterozygous mutation is seen for *N-ras* codon 12, in which the secondary PCR product of 92 bp derived from the normal sequence was digested into products of 73 and 19 bp by *MspI*. A mutation in position 1 or 2 of the codon should destroy the *MspI* site, leading to a digestion-resistant product. As predicted, a G→T transversion at position 1 of the codon led to incomplete *MspI* digestion (Fig. 1). Similarly, for *N-ras* codon 61, the secondary PCR product derived from normal DNA was entirely digested by *MscI*, whereas HT 1080 DNA, containing a heterozygous position 1 C→A transversion, yielded two bands (not shown). DNA from the patient with HCD showed the same patterns as HT 1080 DNA (not shown). A homozygous mutation is shown in *K-ras* codon 12, where the normal secondary product was completely digested, and a homozygous position 2 G→T transversion rendered the PCR product resistant to digestion (Fig. 2). The *K-ras* 12 test consistently demonstrated the most resistance to digestion, and required the greatest excess of endonuclease; why this was so, particularly in comparison to the similar, easily digested *N-ras* test, is unclear. Because of this difficulty, an alternative strategy for this codon has also been developed, using a doubly mismatched primer and *BsaI* (D.R. Jacobson and N.E. Mills, unpublished). PCR products were also obtained using the other seven screening primers listed in Table 1, A and B, and were completely digested by the appropriate restriction enzymes, indicating successful creation of restriction sites (Fig. 3 for KS61S and NS61S, others not shown).

DISCUSSION

We have overcome the fact that no activating *ras* mutation changes a restriction site by using PCR primers containing one- or two-base mismatches from the target sequence to create artificial

TABLE 1A Screening PCR Primers and Restriction Sites Formed

Codon	Screening Primers and Restriction Sites Created		Restriction enzyme
	Name	DNA Sequence	
K-Ras 12	KS12	5'AACTTGTGGTAGTTGGAGCCggt... 10 11 12 / \ / \ / \	Msp I
N-Ras 12	NS12	5'AACTGGTGGTGGTTGGAGCCggt... 10 11 12 / \ / \ / \	Msp I
K-Ras 13	KS13	5'TTGTGGTAGTTCACAGCTGGTggc... 11 12 13 / \ / \ / \	Pflm I
N-Ras 13	NS13	5'TGGTGGTGGTTCCAGCAGGTggg... 11 12 13 / \ / \ / \	Pflm I
K-Ras 61 (CA-)	KS61	5'ATATTCTCGACACAGCTGATcaa... 59 60 61 / \ / \ / \	Bcl I
N-Ras 61 (CA-)	NS61	5'ACATACTGGATACAGCTGGCcaa... 59 60 61 / \ / \ / \	Msc I
K-Ras 61 (--A)	KS61S	5'ATTCTCGACACAGCAGGTTaa... 59 60 61 / \ / \ / \	Mse I
N-Ras 61 (--A)	NS61S	5'ATACTGGATACAGCTGGTTaa... 59 60 61 / \ / \ / \	Mse I

TABLE 1B PCR Primers Used to Determine Whether Mutations at the Codon 61 Position 3 Are Activating (A→C or A→T) or Silent (A→G)

Codon	Screening Primers and Restriction Sites Created		Restriction enzyme
	Name	DNA Sequence	
K-Ras 61 (--G)	KS61G	5'CTCATTGCACTGTACAGTCctg... 63 62 61 / \ / \ / \	Alwn I
N-Ras 61 (--G)	NS61G	5'CTCATGGCACTGTACAGTTCctg... 63 62 61 / \ / \ / \	Alwn I

sites. During PCR, the same substitutions are introduced into both normal and mutated sequences, but restriction sites are formed only in PCR products derived from normal alleles. This strategy was used to demonstrate activating mutations in *N-ras* codons 12 and 61 and in *K-ras* codon 12, and the absence of other activating *ras* mutations in the samples tested. For codons 12 and 13, a mutation in position 1 or 2 eliminates the artificial restriction site for *Msp*I (codon 12) or *Pflm* I (codon 13). Codon 61 screening is performed in two steps because CAA→CAC and CAA→CAT mutations are not silent, and no enzyme can readily test all three positions. To

screen positions 1 and 2, a site ending in CA was created for *Bcl*I (*K-ras*) or *Msc*I (*N-ras*). This step demonstrated the *N-ras* codon 61 mutation in HT 1080 DNA and revealed a mutation in DNA derived from a patient with HCD, suggesting that *ras* activation plays a role in HCD. To screen codon 61 position 3, artificial *Mse*I restriction sites are created. This method detects any mutation in position 3 of codon 61, including the silent transition A→G. The primers in Table 1B are used to distinguish between a silent and activating mutation; the nested (3') primer is used in the secondary PCR with the original 5' primer.

PCR primers containing single-base

FIGURE 1 PCR products amplified using screening primer NS12, *Msp*I-digested. (Lane 1) Control DNA; (lane 2) Molt 4 DNA. Sizes are in base pairs.

3' mismatches have previously yielded products visible on agarose gels, when the mismatched bases retained one purine and one pyrimidine; other mismatches were more destabilizing.^(2,6) Similarly, we used primers containing single mismatches in the 3' base to screen for mutations in *N-ras* codons 12 and 61 and *K-ras* codon 12, and in the penultimate base to screen position 3 of *K-ras* codon 61. Because of the sequences around the other codons of interest, strategies using single mismatched primers were not available. We reasoned that a screening primer containing two mismatches might work in PCR, particularly if the mismatches were not near the 3' base. In four cases (KS13, NS13, KS61G, and NS61G), the two mismatches were three or more bases from the 3' end. The other two screening primers containing double mismatches (KS61 and NS61S) contained mismatches closer to their 3' ends; primer NS61S, which we predicted would be most likely to fail in the PCR, created mismatches between two pyrimidines at both the penultimate and antepenultimate bases; nonetheless, this primer pro-

FIGURE 2 PCR products amplified using screening primer KS12, *Msp*I-digested. (Lane 1) Undigested control DNA; (lane 2) *Msp*I-digested control DNA; (lane 3) undigested SW 480 DNA; (lane 4) *Msp*I-digested SW 480 DNA.

FIGURE 3 PCR products amplified from control DNA using the screening primer NS61S (lanes 1 and 2) or KS61S (lanes 3 and 4), before (lanes 1 and 3) and after (lanes 2 and 4) *MseI* digestion.

vided high yields (Fig. 3), suggesting that the matched 3' base counteracted the adjacent mismatches.

Recently, activating *ras* mutations have been most often detected by allele-specific oligonucleotide (ASO) hybridization,⁽⁷⁾ which requires one labeled oligonucleotide for each possible mutation—40 in all. In the PCR-PIRA method, because each screening primer creates a restriction site in the presence of the normal sequence, each one tests simultaneously for three or six possible mutations; thus, only 10 screening primers are required (although the PCR-PIRA method does not completely define the mutation present). PCR-PIRA is adaptable to the analysis of most point mutations in any gene when no naturally occurring restriction site is present. We demonstrate the feasibility of using primers with two-base mismatches, which makes this method more powerful than if one uses only single-mismatch primers. PCR-PIRA is simple and non-radioactive, and should facilitate the analysis of mutations in *ras* genes and throughout the genome.

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