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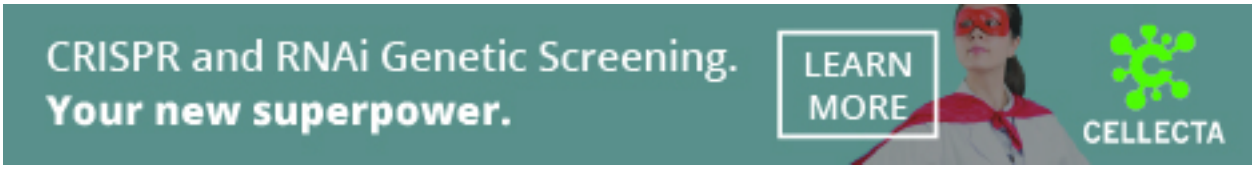

PCR-based Approaches for Detection of Mutated *ras* Genes

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The *ras* genes encode 21-kD GTP-binding proteins ($p21^{ras}$) that cycle between an inactive GDP-bound form and an active GTP-bound form. The $p21^{ras}$ proteins are thought to play an important role in signal transduction that eventually controls proliferation and differentiation of many different cells. The *ras*(proto-onco)genes can be activated as oncogenes by simple point mutations, which substitute a single amino acid residue at a critical position in the protein product. Mutated *ras* genes occur with a high frequency in common human cancers, including adenocarcinoma of the lung, colorectal cancer, myeloid leukemia, and adenocarcinoma of the pancreas.(1)

Originally, the transforming *ras* mutations were determined by transfection of NIH-3T3 cells, followed by cloning and sequencing of the oncogene. The mutations found are almost without exception point mutations at codons 12, 13, or 61 of the highly homologous *ras* genes H-*ras*, K-*ras*, and N-*ras*. The introduction of the polymerase chain reaction (PCR) technique has greatly simplified the procedure for analyzing tumors for the presence of one of these point mutations. A source for the DNA to be analyzed may be either fresh or frozen tissue samples. Also formalin-fixed, paraffin-embedded tissue sections may be used as starting material, allowing retrospective analysis of archival material. Different methods have been devised to detect the common point mutations in the PCR-amplified *ras* gene segments, as shown in Table 1. We will discuss these different methods in this review.

Allele-specific Oligonucleotide Hybridization

Allele-specific oligonucleotide hybridization (ASO) to detect point mutations is based on the fact that an oligonucleotide with one mismatch binds more weakly to its (imperfect) complement than a perfectly matching one. By performing hybridization and washing just below the dissociation temperature (T_m) of the perfectly matching oligonucleotide, one can discriminate between a perfectly matching oligonucleotide and an oligonucleotide with 1-bp mismatch.(2) In short, PCR-amplified DNA is spotted onto a nylon (or nitrocellulose) filter and hybridized to a radioactively labeled oligonucleotide which corresponds to the wild-type sequence of the particular *ras* codons. The filter is washed under stringent conditions and hybridization is detected by autoradiography. This procedure detects the presence of the wild-type allele of the gene segment. Duplicate filters are then hybridized with different, labeled oligonucleotides, each of which corresponds to one of the expected point mutations in the gene segment. Hybridization of one of these allele-specific (point mutation-specific) oligonucleotides shows the presence of the corresponding point mutation. Hybridization and stringent washing may be performed in the presence of 3 M tetramethylammonium chloride.(3) At this concentration the G-C and A-T base pairs have similar stabilities, so that oligonucleotides of the same length have the same T_m, regardless of the base composition. This allows hybridization and washing of the filters with different oligonucleotides next to each other in one procedure. Using this method, large series of DNA samples can be analyzed in a short period of time for the presence of anticipated point mutations. This method has been applied in numerous studies in which tumors were examined for the presence of *ras* gene mutations.(1)

TABLE 1 Detection of Common Point Mutations in PCR-amplified *ras* Gene Segment

Tissue→DNA→PCR	Nature of the mutation (+/−)[a]
Allele-specific oligonucleotide hybridization	+
RNase mismatch cleavage	−
Single-strand conformation polymorphism	−
DNA sequence analysis	+
(Primer-mediated) RFLP	−/+
Mutant-enriched PCR	−
Allele-specific PCR	+

[a]The minus indicates that additional analysis is required to determine the nature of the mutation. See text for references.

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Recently, a reverse oligonucleotide hybridization method has been developed.[4] Oligonucleotide probes are immobilized on nylon filters through a covalent binding reaction and PCR products are hybridized to the oligonucleotide probes. PCR products were labeled with biotin using biotinylated primer oligonucleotides. In combination with a chemiluminescent detection system, this yields a very sensitive nonradioactive procedure. This approach allows the simultaneous hybridization of one PCR-amplified DNA sample to a large number of different oligonucleotide probes.

RNase Mismatch Cleavage

The RNase mismatch cleavage procedure can detect the position of a mutation in the target DNA.[5,6] The method involves hybridization of a radioactively labeled wild-type RNA probe to PCR-amplified DNA, which may contain a mutation. RNase A will cleave the RNA at the position of a mismatch. The resulting RNA fragments are analyzed by gel electrophoresis and the lengths of the fragments give the position of the mutation. Certain mismatches in the DNA : RNA heteroduplexes are quite RNase-resistant,[5,6] requiring that the complementary strands, which give the RNase-sensitive mismatch pair, also be analyzed. Further analysis is required to establish the nature of the mutation. Winter et al.[6] have developed this method to study the amplification and expression of the human mutant K-*ras* gene, using DNA and RNA isolated from tumor cell lines. Almoguera et al.[7] and Perucho et al.[8] have applied this method to PCR-amplified DNA from formalin-fixed, paraffin-embedded samples from biopsies, surgical resections, and autopsies.

Single-Strand Conformation Polymorphism

Hayashi and co-workers[9–11] have devised a method, single-strand conformation polymorphism (SSCP), to detect mutations in DNA that is based on conformation polymorphism of the separated single strands of PCR products. First, the target sequence in genomic DNA or cDNA is amplified and simultaneously labeled using radioactively labeled PCR primers or dNTPs. Subsequently, the PCR product is denatured and analyzed by electrophoresis on a non-denaturing polyacrylamide gel. Mutations are detected by altered mobility of the separated strands relative to the strands of the normal, wild-type PCR product. This approach shows structural alterations, including point mutations in the PCR products. This method has been used to study *ras* gene mutations in human lung cancer.[12] It was shown that the 10 different point mutations examined after amplification in 100- to 200-bp fragments could all be detected by significant mobility shifts in at least one of the separated strands. The mutations can be elucidated by isolation of each polymorphic DNA band from the gel and amplification by a second PCR for sequence determination. By this technique, the sequence of a minor constituent (approximately 3%) can be determined accurately.[13] A nonradioactive SSCP is described by Ainsworth et al.,[14] who performed an asymmetric amplification on PCR-amplified products and detected polymorphic DNA products by silver staining.

DNA Sequence Analysis

Sequence analysis of PCR-amplified DNA represents the most definitive method for the identification of (point) mutations. Also, mutations in the amplified segment that had not been anticipated can be detected. However, the mutant sequence can only be read in the combined wild-type and mutant sequence ladder if the mutant allele is present at a certain minimal percentage. Methods that detect mutations but not the exact nature of the mutation [for example SSCP and restriction fragment length polymorphism (RFLP) analysis (see below)] frequently use characterization of the mutation by sequence analysis. Sequence analysis of PCR-amplified DNA has been used widely to study *ras* gene mutations in different tumors,[15–18] and different approaches (see, for example, refs. 15, 19, and 20) have been followed. A method by which gel-purified PCR products can be sequenced directly[21] allows easy and fast characterization of products of RFLP analyses.

Important for clinical use are methods that facilitate the linking of enzymatic amplification to automatic sequencing. This can be reached by incorporation of biotin in one of the amplification primers. The PCR products can be isolated easily using avidin– or streptavidin–agarose or magnetic beads coated with streptavidin.[22,23] The two strands of the PCR products can then be separated by denaturation with alkali and sequenced. Shaw et al.[24] have applied this approach to study K-*ras* mutations in primary colon tumors.

Syvänen et al.[25] have developed a solid-phase minisequencing method. The PCR-amplified DNA fragment is immobilized using a biotinylated primer and streptavidin-coated magnetic beads. The nonbiotinylated strand is removed by denaturation with alkali. Mutations are identified in primer extension reactions with a primer that has its 3′ end next to the position where a mutation can occur. Four separate primer extension reactions, each with one of the four radiolabeled dNTPs, are performed. Incorporation of a particular nucleotide then shows the presence of its complement in the immobilized template strand. The method has been used to study N-*ras* mutations in acute myeloid leukemia and has identified a mutant *ras* allele in a sample that consisted of 99.7% normal cells.

Multiplex PCR amplification and direct sequencing of the homologous *ras* sequences have been described by Manam and Nichols.[26] In this procedure, the regions surrounding codons 12–13 and 61 of the three *ras* genes are amplified simultaneously. Each of the amplified *ras* segments in this mixture is then sequenced separately using high-stringency annealing and extension of the specific sequence primer permitted by *Taq* polymerase.

Restriction Fragment Length Polymorphism

The presence of a point mutation in a certain DNA segment can create or destroy a restriction enzyme recognition site and thus result in an RFLP. For example, the polymorphism for the enzyme *Hpa*II, which is the result of any change in the first or the second nucleotide of codon 12 of H-*ras*,[27] has been used to detect point mutations at

these positions in PCR-amplified DNA.[28] Analysis of the PCR products after restriction enzyme incubation, gel electrophoresis, and staining with ethidium bromide directly shows whether the anticipated point mutation is present or not. Nakazawa et al.[29] have developed a sensitive method to detect an A to T mutation at codon 61 of H-*ras*; this transversion results in an *Xba*I cleavage site. PCR amplification was performed using radioactively labeled oligonucleotide primers and T7 DNA polymerase. Presence of the radioactive fragment which corresponds to cleavage by *Xba*I at codon 61 is diagnostic for the mutation. Using radioactive detection, mutant alleles can be detected at a frequency of $1:10^6$ wild-type alleles. T7 DNA polymerase was used because errors generated by *Taq* polymerase created the *Xba*I cleavage site at a frequency that interferes with the sensitivity that was reached. T7 DNA polymerase is presumably more accurate than *Taq* polymerase.[20]

Primers that contain mismatches can be used efficiently for PCR amplification. This opens the possibility for creating (or destroying) restriction enzyme recognition sites in PCR-amplified DNA. Also mismatch primers that yield (or destroy) a restriction enzyme recognition site in combination with the nucleotide sequence in the target DNA immediately downstream from the primer can be used. Point mutations at the specific sites in the target DNA then destroy (or create) the restriction enzyme recognition site and can thus be detected by restriction enzyme incubation and gel electrophoresis. Jiang et al.[30] have used this mismatch primer approach to create a *Bst*NI polymorphism for mutations at positions 1 and 2 of codon 12 of K-*ras* (the mutant DNAs are not cleaved). Kumar and colleagues[31,32] have developed a sensitive method to detect G to A mutations at position 2 of codon 12 of H-*ras*. An *Xmn*I recognition site is created by the mutated codon 12 and mismatches in the 3′ oligonucleotide primer. After incubation with *Xmn*I, the amplified wild-type and mutant DNA fragments are detected by hybridization to a radioactive probe, followed by a gel retardation assay. Using this procedure, a *ras* oncogene can be identified in a single cell mixed up with 10,000 normal cells.[31] Mitsudomi and colleagues[33] have devised a large number of mismatch oligonucleotide primers that generate, in combination with different, specific *ras* gene point mutations, new restriction sites. Similarly, Jacobson and Moskovits[34] have devised a set of primers that generate RFLPs for most of the known *ras* point mutations. In this approach the wild-type sequence is cleaved and the mutant sequence remains intact. The mismatch may be located near the 3′ end of one of the PCR primers, or can even be the 3′ nucleotide of a PCR primer,[34,35] although it has also been reported that a mismatch at the 3′ end of a primer stops the amplification process.[36]

The nature of the point mutation detected by this primer-mediated RFLP analysis will be (mostly) known if a new cleavage site is generated by the mutation.[33] Loss of the cleavage site requires additional analysis (ASO or sequence analysis of the restriction enzyme resistant PCR product) to determine the nature of the change in the DNA.

Mutant-enriched PCR Amplification

Recently, a primer-mediated RFLP method with enhanced sensitivity for the detection of mutant K-*ras* alleles has been devised.[37–39] The high sensitivity is achieved by selective PCR of mutant K-*ras* gene sequences using a two-stage procedure (Fig. 1). First the DNA is amplified using a (mismatch) primer that generates a restriction enzyme recognition site only in combination with the wild-type sequence. Incubation with the restriction enzyme cleaves the amplified wild-type sequences and leaves the mutant sequences intact. Subsequently, the noncleaved mutant sequences are amplified in a second PCR using the same (mismatch) primer, incubated again

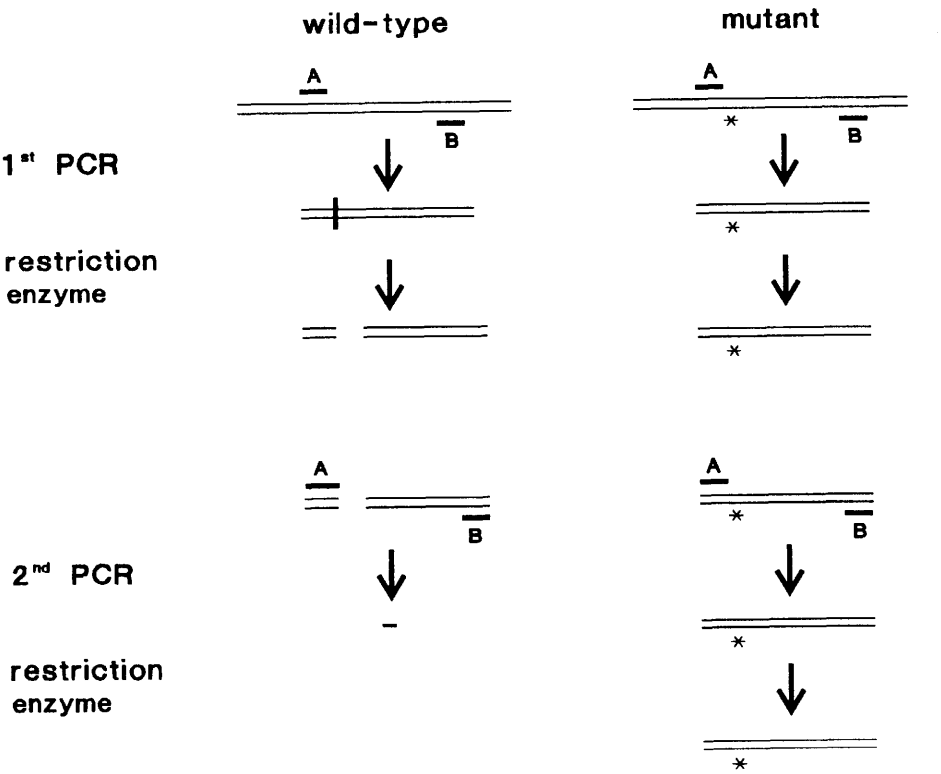

FIGURE 1 Mutant-enriched PCR amplification. The DNA, which may contain a mutation (*), is amplified using primers A and B (horizontal bars). Primer A may have 1- or 2-bp mismatches, which generate a restriction enzyme recognition site (vertical bar) in the wild-type allele but not in the mutant allele. Incubation with the restriction enzyme cleaves the amplified wild-type sequence and leaves mutant sequence intact. The same primers A and B are used in a second PCR, and now only the contiguous, uncleaved mutant sequence is amplified. The products of the second PCR are incubated with the restriction enzyme. The restriction enzyme-resistant DNA fragment is diagnostic for the presence of a mutant.

with the restriction enzyme, and analyzed for RFLP. Restriction enzyme-resistant PCR product, which can be detected by gel electrophoresis, is diagnostic for the presence of a mutation at the anticipated site. The nature of the mutant can then be determined by direct sequencing of the restriction enzyme-resistant material or by ASO of the mutant-enriched material obtained after the second amplification reaction (unpubl.). The sensitivity of this procedure is limited by the fidelity of the polymerase and the method of detection. However, if the number of cycles is kept low (12–15) in the first PCR of the procedure, a *ras* mutation occurring in 1 per 1000 cells can be detected by staining with ethidium bromide without detecting mutations introduced by *Taq* DNA polymerase during PCR.[37–39]

Allele-specific PCR

Allele-specific PCR is based on the exact complementarity between the template DNA and the primer. Templates that match the primer perfectly at the 3′ terminal nucleotide are amplified efficiently, whereas no amplification is detected when there is a single mismatch at this position.[36,40,41] Thus, allele-specific PCR can be performed using oligonucleotides whose 3′ terminal nucleotides correspond to specific point mutations. Ehlen and Dubeau[36] and Stork et al.[42] have used this approach successfully to detect *ras* mutations in cell lines and pancreas tumors. However, the conditions under which these allele-specific PCRs are performed are very critical, as shown by Kwok et al.[41] and as may be concluded from the observations of Haliassos[35] and Jacobson and Moskovits,[34] who amplified *ras* gene segments efficiently using an oligonucleotide with a mismatch at the 3′ terminal nucleotide.

DISCUSSION

Mutations in one of the three *ras* genes are one of the most frequently occurring genetic events in human carcinogenesis known today, only outranked by mutations in the p53 gene. Therefore, the detection of mutations in *ras* genes will be a recurring analysis in a large number of laboratories. First, the detection of *ras* point mutations may be of prognostic or diagnostic significance. In this respect, the study of Rodenhuis and co-workers[43] is worth mentioning because it shows that the presence of a mutation in the K-*ras* gene in adenocarcinoma of the lung is a marker for poor prognosis. Second, the characterization of genetic events underlying the development of a particular tumor may need the analysis of *ras* point mutations. Third, mutations in *ras* genes may be a useful marker for determining residual disease in leukemia, and, finally, mutations in *ras* genes may be indicative for the involvement of carcinogens.[1]

Not all procedures described in this review, however, are suitable for routine analysis, and the most appropriate procedure depends on the tumor type and the information necessary. For instance, *ras* point mutations in adenocarcinoma of the pancreas and the lung occur almost exclusively in codon 12 of the K-*ras* gene. To detect these mutations, the most useful method is the restriction fragment length polymorphism (RFLP) analysis on mutant-enriched PCR material (Fig. 2). This method is easy, fast, and reliable. Furthermore, it does not need radioactively labeled reagents, and is therefore very suitable for use in clinical laboratories. Finally, the method is very sensitive, allowing the detection of mutations in a background of normal tissue. In principle, this method can be used for most of the codons where mutations occur (see, for instance, ref. 34). This procedure, however, does not elude the exact mutation in the codon, information not necessary for the evaluation of the genetic events underlying tumor formation. RFLP analysis on mutant-enriched PCR material in combination with ASO may be the method of choice to determine the exact mutation.

DNA sequence analysis methods are very reliable procedures, providing that the majority of the tissue consists of tumor cells. The microsequencing procedure of Syvänen et al.[25] allows detection of a mutation in a high background of wild-type sequences and sequence analysis of mutant-enriched PCR material is very sensitive as well. The RNase mismatch procedure and especially the SSCP procedure are more suitable for screening for mutations that can be located at different positions in a DNA segment, such as the mutations occurring in the p53 gene. For such analyses, direct sequencing is also very suitable and the method most used.

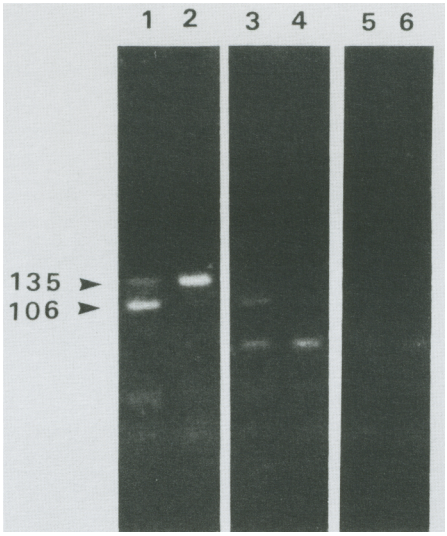

FIGURE 2 RFLP analysis of mutant-enriched material (see also Fig. 1). K-*ras* DNA was amplified as described by Levi et al.[35] Prior to the second PCR, samples were incubated with *Bst*NI (even numbers) or not (odd numbers). After the second PCR, all samples were incubated with *Bst*NI and analzyed by agarose gel electrophoresis in the presence of ethidium bromide. The positions of *Bst*NI-resistant (135) and *Bst*NI-cleaved (106) PCR products are indicated. The faster-migrating bands probably represent primer oligomers. (Lanes *1* and *2*) Template DNA from a tumor containing a K-*ras* codon 12 mutation; (lanes *3* and *4*) template DNA from a tumor which does not contain a K-*ras* codon 12 mutation; (lanes *5* and *6*) no template DNA added.

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