

Detection of *K-ras* Oncogene Mutations by Polymerase Chain Reaction-Based Ligase Chain Reaction¹

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To evaluate a rapid multiplexed assay to detect three common *K-ras* codon 12 mutations, primer pairs complementary to the wild-type and mutant loci were developed and tested with lung cancer cell lines with previously identified mutation status. The sensitivity of detection of mutations was determined to be at least 1% using spiked samples containing *K-ras* codon 12 mutations. This assay was then used to evaluate prospectively *K-ras* status in airways of individuals at high risk of lung cancer by analysis of bronchoalveolar lavage (BAL) specimens from patients who have been previously treated for lung cancer. DNA was extracted from BAL specimen cell pellets, and PCR-based ligase chain reaction was performed for mutations in the first position of codon 12 of *K-ras*, with positive and negative controls. Of 10 BAL samples, 4 contained 1 mutation (GGT → TGT), 1 contained 2 mutations (GGT → TGT and GGT → AGT), and the rest were wild-type. The BAL mutations were validated by cloning and screening with mutant-specific probes followed by confirmation sequencing. © 1996 Academic Press, Inc.

Mutation at specific codons in protooncogenes is a well-studied event in their activation. About 80 to 90% of activating mutations of the *K-ras* gene occur at codon 12 or 13 in human non-small cell lung cancer and in colorectal cancer, most of which are in the first position of codon 12 (1). An approach was developed by Sidransky and co-workers to determine if a *ras* mutation was present in a primary tumor, and then screen for that

particular *ras* mutation to identify if the mutation was detectable in sputum at an earlier stage (2). However, this approach is not feasible for early detection of cancer in clinical samples. Therefore, methods are needed to screen rapidly for multiple mutations at *K-ras* codon 12 using premalignant clinical specimens in which a very small percentage of mutated cells may be present. In developing an assay for population-based early cancer detection, the clinically relevant goal is not a rapid assay to screen tumors for all possible mutations, but rather the identification of clonally expanded populations of exfoliated epithelial cells bearing critical *K-ras* mutations.

A number of amplification methods have been developed to determine single base mutations. These methods fall into two main categories. The first category of methods takes advantage of existing or introduces new restriction sites into PCR products at the mutated codons. The second category consists of amplification-based techniques in which PCR product synthesis or detection of PCR products depends on mutation-specific design of oligonucleotide primers and probes. This category includes the ligase chain reaction (3) which utilizes DNA ligation of adjacent mutant and/or wild-type primers to identify the presence or absence of single base mutations.

We chose to apply the ligase chain reaction (LCR)³ in conjunction with PCR as a technique for determination of single base variants of the *K-ras* codon 12 mutation. A schematic representation of this PCR-based LCR assay is provided (see Fig. 1). This methodology has greater potential for rapid and automated analysis compared to presently available methods. Previously, the LCR methodology has been utilized for the detec-

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³ Abbreviations used: LCR, ligase chain reaction; BAL, bronchoalveolar lavage.

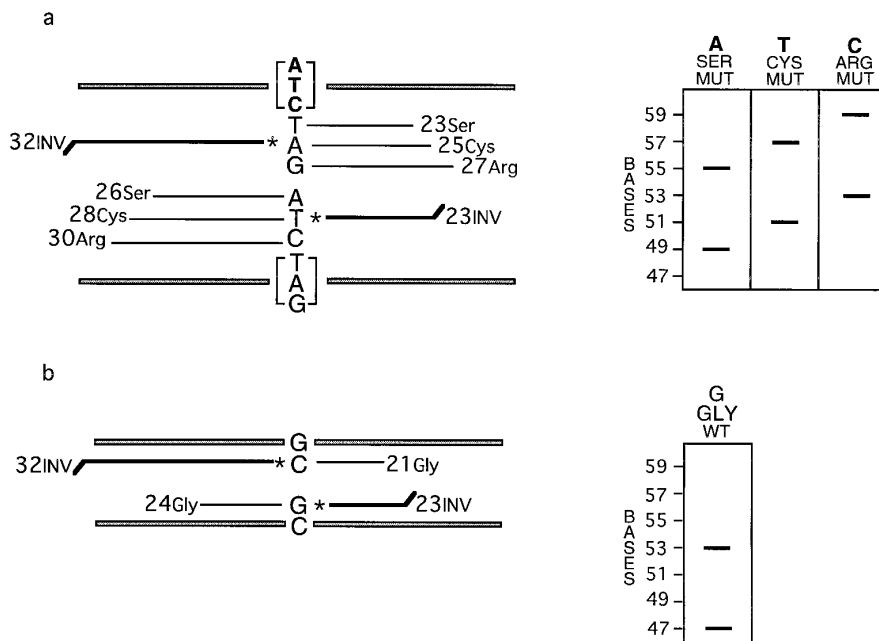


FIG. 1. Schematic representation of PCR-based LCR assay strategy and primer design for determination of *K-ras* codon 12 mutations. (a) A set of eight primers including six diagnostic primers and two invariant labeled primers are used in the LCR reaction mix for the determination of A, T, and/or C mutation (MUT) in the first base of *K-ras* codon 12. Diagnostic primer pairs are designated by their size and target base (23Ser and 26Ser for the A mutation, 25Cys and 28Cys for the T mutation, and 27Arg and 30Arg for the C mutation) and represented schematically on the left panel. Gel analysis of LCR products of this reaction of each pair of diagnostic primers with the invariant primers 32INV and 23INV (bold lines) results in the formation of the band patterns shown in the right panel of a. (b) A schematic diagram illustrating the use of the wild-type primers 21Gly and 24Gly with the invariant primers 32INV and 23INV in a separate LCR reaction. Gel analysis of the LCR products allows the detection of the presence of the wild-type base G in the first position of *K-ras* codon 12 as two bands of 47 and 53 bases shown in the right panel.

tion of a variety of genetic diseases involving point mutations (for example, sickle cell anemia and cystic fibrosis), and for detection and identification of bacteria and viruses in human samples. These current areas of application of LCR were reviewed (4). However, application of this technology for *ras* mutations has not been reported, except for codon 61 of the mouse *Ha-ras* gene (5). By appropriate primer design, as reported here, LCR may be applied to the simultaneous detection of three mutations at the first base of *K-ras* codon 12. The accuracy of the PCR/LCR assay was validated with DNA from cell lines with known *K-ras* mutations. The *K-ras* LCR assay was used to determine mutations present in cells derived from bronchial washings from previously resected non-small cell lung cancer patients. The results were confirmed by oligonucleotide-specific hybridization and sequence analysis.

MATERIALS AND METHODS

Positive Control Cell Lines

Cell lines were obtained with previously published *K-ras* codon 12 sequences. HeLa cell line was utilized for wild-type control (GGT^{Gly}), Calu 1 for mutant T

(TGT^{Cys}) (6, 7), A549 for mutant A (AGT^{Ser}) (8), and H157 for mutant C (CGT^{Arg}) (9).

Bronchoalveolar Lavage Specimens

Bronchoalveolar lavage (BAL) specimens in normal saline containing protease inhibitors were obtained from Stage I, resected, non-small cell lung cancer patients by a previously described procedure (10, 11). The specimens were collected at clinical centers of the Lung Cancer Early Detection Working Group (LCEDWG) and shipped to the Biomarkers and Prevention Research Branch on ice. Cell pellets of approximately 1×10^6 cells were aliquoted from each specimen and frozen at -80°C for extraction of DNA.

DNA Isolation

DNA was extracted from cell lines and BAL samples using standard procedures (12). Cell pellets were resuspended in $1 \times$ proteinase K digestion buffer (10 mM Tris-HCl, pH 7.8, 5 mM EDTA, 0.5% SDS, 200 $\mu\text{g/ml}$ proteinase K) and incubated overnight at 56°C . Following phenol and phenol/chloroform extraction, the DNA was precipitated and pelleted. DNA pellets were resuspended in sterile water and quantitated by A_{260}/A_{280} .

TABLE 1

Sequences of Wild-Type and Diagnostic LCR Primers

Primer	Bases	Sequence (5' → 3')
Ser ^{AGT} -C (26Ser)	26	GATATTTCTTGTGGTAGTTGGAGCTA
Ser ^{AGT} -NC (23Ser)	23	TTAAAGCTCTTGCCTACGCCACT
Cys ^{TGT} -C (28Cys)	28	ATGATATAACTTGTGGTAGTTGGAGCTT
Cys ^{TGT} -NC (25Cys)	25	AATTAATAACTCTTGCCTACGCCACA
Arg ^{CGT} -C (30Arg)	30	AAATGATATAACTTGTGGTAGTTGGAGCTC
Arg ^{CGT} -NC (27Arg)	27	AAAATTAATAACTCTTGCCTACGCCACC
Gly ^{GGT} -C (24Gly)	24	TAAAACCTTGTGGTAGTTGGAGCTG
Gly ^{GGT} -NC (21Gly)	21	AACACTCTTGCCTACGCCACC
Invar-C (23INV)	23	GTGGCGTAGGCCAAGAGTGCCTTG
Invar-NC (32INV)	32	AGCTCCAACCTACCACAAGTTTATATTCAGTCA

PCR Conditions

The region surrounding codon 12 of K-*ras* was amplified in order to increase the number of target sequences for the ligase chain reaction assay. The following amplification primers were used to generate a 115-bp product: RS53 (5'-CCTGCTGAAAATGACTGAAT-3') and RS54 (5'-TGTTGGATCATATTCGTCCA-3') (13). Each 50- μ l reaction contained the following: 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 2.5 mM MgCl₂, 0.2 mM each dNTP, 25 pmol RS53, 25 pmol RS54, 2.5 units *Taq* DNA polymerase and 100 ng template DNA (purified human DNA). The samples were amplified for 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 30 s.

Ligase Chain Reaction of PCR-Amplified DNA

A multiplex LCR was performed to determine the first base of codon 12 of the K-*ras* gene. Following amplification, 1 μ l of each PCR product was added to a master mix of six mutant primers and two ³²P-labeled invariable primers. All primers used for LCR were OPC purified (see Table 1 for primer sequences). The primers were designed so that the respective lengths of the sense and antisense for the Gly^{GGT} ligated DNA are 47 and 53 bp, the Ser^{AGT} ligation products are 49 and 55 bp, the Cys^{TGT} products are 51 and 57 bp, and the Arg^{CGT}-ligated DNAs are 53 bp and 59 bp. The differences between products ranging from 47 to 59 bp are sufficient for differentiation using denaturing 10% polyacrylamide gels. In several primers, mismatched bases were incorporated to alter the *T_m*s. To ensure that the primers were ligating correctly, all primers were empirically tested using PCR-based LCR of known mutant PCR products. Each LCR result was confirmed at least twice using two independent PCR reactions to ensure that neither *Taq* DNA polymerase nor *Taq* DNA ligase errors had occurred at the base of interest.

Several modifications of previously described LCR conditions (3) were utilized, including the substitution of 1 μ l of PCR product for genomic DNA. The unphos-

phorylated Invariant C (coding) and Invariant NC (noncoding) primers were 5' α -³²P-endlabeled using T4 polynucleotide kinase. A mixture of the six unlabeled diagnostic primers and the two labeled invariant primers was utilized for each diagnostic LCR reaction. Each 10- μ l LCR reaction mixture contained the following: 20 mM Tris-HCl (pH 7.6), 25 mM potassium acetate, 10 mM magnesium acetate, 10 mM DTT, 1 mM NAD⁺, 0.1% Triton X-100, 0.4 μ g salmon sperm DNA, 15 nick closing units *Taq* DNA ligase (New England Biolabs, Beverly, MA), 40 fmol each diagnostic primer, 40 fmol each α -³²P-endlabeled invariant primer ($\approx 4 \times 10^5$ cpm), and approximately 1 fmol template DNA. LCR reactions were overlaid with mineral oil and incubated at 94°C for 2.5 min followed by 94°C for 1 min and 65°C for 4 min for 30 cycles. At completion of LCR, 2 μ l loading dye and 4 μ l of each reaction were heated at 94°C for 5 min, and chilled on ice prior to gel analysis. The samples were analyzed by denaturing gel electrophoresis using a 10% polyacrylamide gel containing 7 M urea. The LCR products as well as the unligated primers were detected by exposure of the dried gel to film.

The initial experiments utilized DNA from three different purified cell lines which have been previously characterized for K-*ras* mutations. PCR-amplified DNA from each of the particular cell lines was assayed for K-*ras* codon 12 status using the diagnostic primers in a "multiplex" LCR assay as described above. Similar experiments were performed using identical conditions with the substitution of the two wild-type primers for the set of six diagnostic primers to confirm the presence of wild-type PCR product.

TA Cloning

BAL K-*ras* PCR products were cloned into the TA vector (Invitrogen, San Diego, CA) as recommended by the supplier. Ligations were transformed using electroporation and cultures plated on LB-Amp, X-gal agarose plates. The total number of recombinant clones were determined by blue-white screening.

Screening of Clones

Clones were transferred to nylon membranes, alkali-denatured, neutralized, cross-linked, and prehybridized with Hybrisol I (Oncor, Gaithersburg, MD). Wild-type K-*ras* (Gly^{GGT}-12) and mutant-specific oligonucleotides (Cys^{TGT}-12, Ser^{AGT}-12) (2) were synthesized and end-labeled. Filters were hybridized with either wild-type or mutant probe (1×10^6 cpm) overnight and then washed at a final stringency of 3 \times SSC + 0.1% SDS for 30 min at 54°C. Filters were exposed for 4 h to detect positive clones.

Sequencing of Clones

Colonies corresponding to positive hybridization signals were isolated and grown overnight. DNA was iso-

lated by miniprep procedure, and was used as template for sequencing with SP6 and/or T7 primers using Sequenase chemistry (U.S. Biochem, Cleveland, OH). Results were verified using dye-deoxy chemistry and an automated sequencer (Applied Biosystems, Foster City, CA).

RESULTS

PCR and LCR conditions were first established using lung cancer cell lines with previously characterized *K-ras* first base mutations (GGT → XGT). Only PCRs containing 100% wild-type or 100% mutant codon 12 *K-ras* DNA were used to develop the assay. A number of positive and negative controls were utilized in both PCR and LCR reactions. One or more PCR-negative controls (water only) were included with each set of PCR reactions. As an LCR-negative control, all PCR-negative controls were assayed for presence of wild-type LCR products in each set of LCR assays. HeLa DNA was utilized as an LCR-positive control for presence of wild-type *K-ras* codon 12 product in all LCR assays. After establishing optimum LCR conditions with pure mutant and pure wild-type DNA, PCR reaction mixes in which 10 ng of each mutant DNA was mixed with 90 ng of HeLa DNA prior to PCR were routinely used in all diagnostic LCRs as positive controls for the presence of mutations. Each PCR was tested separately for presence of *K-ras* mutations using diagnostic primers in the LCR, and for the presence of wild-type *K-ras* PCR product using wild-type primers in the LCR.

To determine the sensitivity of detection of *K-ras* codon 12 mutations by PCR-based LCR, two different types of assays were performed. First, each of the mutant DNAs was diluted with wild-type DNA prior to PCR to simulate DNA obtained from a small number of mutant cells in a population of wild-type cells. The ligated products of DNA from each of the mutant cell line were consistently detected at levels of 1% mutant, and occasionally detected at 0.1% mutant DNA (1 ng mutant DNA mixed with 99 ng wild-type DNA, or 0.1 ng mixed with 99.9 ng wild-type DNA, respectively) (see Fig. 2). In the second approach, wild-type or mutant PCR products were serially diluted 1:10 in water, 1 μ l of each dilution was assayed by LCR. Both mixing wild-type and mutant DNA prior to PCR and dilution of pure mutant product with water prior to LCR resulted in at least 1% level of detection for both mutant and wild-type DNA (not shown).

DNAs from the various mutant cell lines were mixed prior to PCR to simulate multiple *K-ras* mutations in a single sample. These mixing experiments demonstrated that simultaneous detection of multiple mutations was possible (see Fig. 3). As a negative control for the LCR reactions, 1 μ l of the PCR-negative control

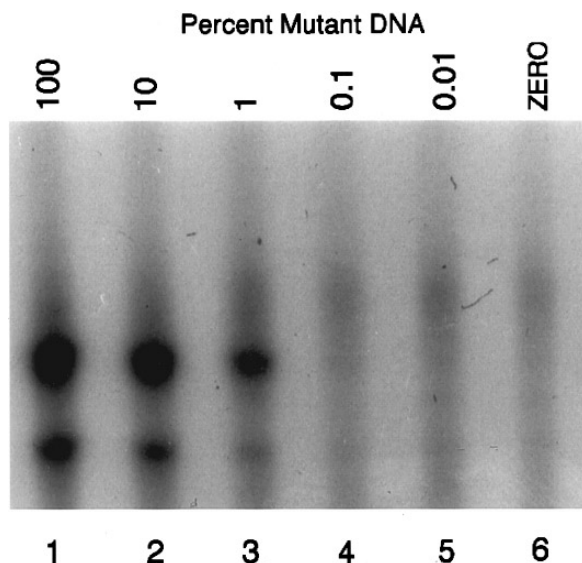


FIG. 2. Dilution experiment demonstrating detection of mutation at level of at least 1% mutant sequence. To determine the sensitivity of detection of *K-ras* codon 12 mutations by PCR-based LCR, *K-ras* codon 12 mutant DNA was separately diluted with wild-type DNA prior to PCR to simulate DNA obtained from a small number of mutant cells in a population of wild-type cells. The PCRs were performed, and 1 μ l of each PCR was used in separate LCR reactions. LCR reactions of undiluted mutant DNA (lane 1), and 10% mutant + 90% wild-type DNA (lane 2), and 1% mutant + 99% wild-type DNA (lane 3) consistently produced the expected results. Mutations present at 0.1% were occasionally detected (lane 4). Detection of mutations at the 0.01% level was not possible using these methods (lane 5). Wild-type DNA alone was used in the PCR reaction as a negative control for mutation analysis. The resulting LCR produced no mutant bands (lane 6).

was used as a template for LCR reactions performed with wild-type LCR primers.

To apply this method to samples with clinical relevance, bronchoalveolar lavage specimens were obtained from Stage I, resected, non-small cell lung cancer patients. BAL fluid is a clinically useful specimen to examine for new early detection markers for lung cancer because it allows sampling of the field of carcinogenesis within the bronchial epithelium. The LCEDWG patients are expected to develop second primary lung cancer at a much higher rate than even a heavy smoking population (10, 11). The samples were encoded to ensure unbiased interpretation of the data.

DNA was isolated from a small fraction of the sample, and PCR-based LCR was performed using the methods and controls described above. Of 10 samples tested, 4 contained 1 mutation (GGT → TGT), 1 contained 2 mutations (GGT → TGT and GGT → AGT), and the rest were wild-type (see Fig. 4). These results were verified by at least two independent PCR reactions followed by LCR. BAL clinical samples have been demonstrated recently by the PCR-PIREMA tech-

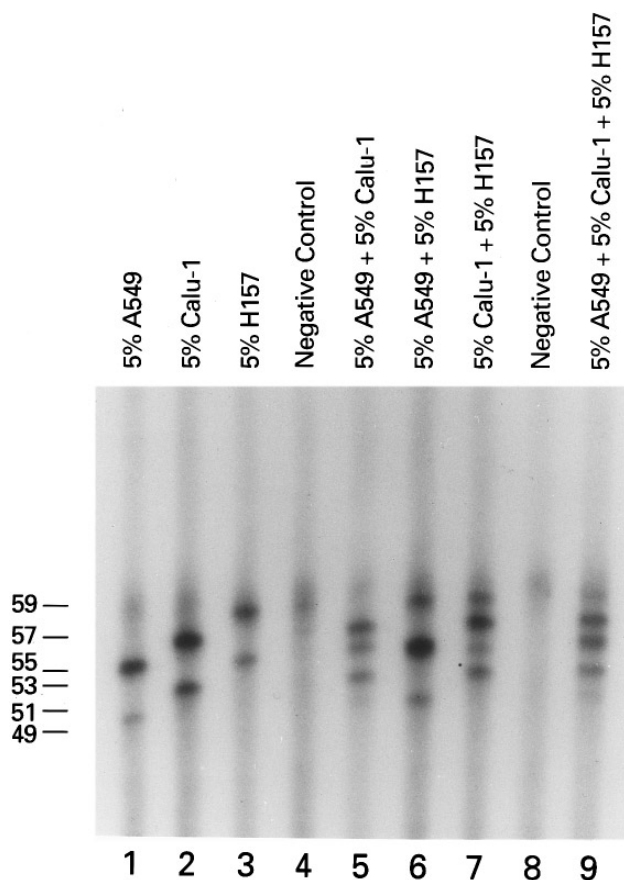


FIG. 3. Mixing experiment for each of the mutations with 5% mutant DNA and determination of two and three mutations together. DNAs from each of the mutant cell lines were tested separately (lanes 1, 2, and 3) and mixed (lanes 5, 6, 7, and 9) prior to PCR to simulate multiple *K-ras* mutations in a single sample. After PCR, 1 μ l of each reaction was used in the LCR reaction with diagnostic primers. These mixing experiments demonstrated that simultaneous detection of multiple mutations was possible. As negative controls for the LCR reactions, 1 μ l of the PCR negative control was used as a template for LCR reactions performed with wild-type LCR primers (lanes 4 and 8).

nique to contain small numbers of cells with *K-ras* mutations (14). The BAL samples were analyzed in parallel with each of the mutant and wild-type positive controls and negative controls.

All BAL PCR samples which did not produce mutant results were analyzed by LCR with wild-type primers to ensure that the ligation reactions were successful. The wild-type ligation products of 47 and 53 bases were detected in each of the five samples, demonstrating that the negative analysis for a mutation was not due to lack of PCR amplification, or inhibition of the LCR reaction (not shown).

In order to validate the mutations produced by the LCR procedure, we utilized a modification of the cloning/mutant specific screening technique described by

Sidransky and co-workers (2). Each of the PCR reactions was cloned using the TA vector technology, and bacteria were transformed with the ligated product via electroporation and plated. Colony lifts were made, and the filters were probed with both wild-type and mutant-specific probes for *K-ras* as described (2). Approximately 1% of the colonies on each plate produced a signal when hybridized with the mutant-specific probe (5 signals from approximately 500 colonies). Colonies corresponding to the signals were isolated and expanded and DNA was extracted from each for confirmation sequencing of the *K-ras* codon 12 region. Each mutation detected by LCR was confirmed by manual sequencing in both directions, and also by automated sequencing using ABI automated sequencer (not shown). This included four BAL samples which had GGT \rightarrow TGT mutations, and one BAL sample which was probed separately using two different mutant probes and produced both GGT \rightarrow TGT and GGT \rightarrow AGT mutations upon sequencing.

DISCUSSION

The PCR/LCR method presented in this paper is a sensitive and rapid assay which can detect low levels of *K-ras* mutations in clinical specimens. An important application of this tool is the detection of genetic lesions which may be present in only a small number of cells. Mixing and dilution experiments demonstrated that detection of a *K-ras* mutation is possible at levels of at least 1% mutant in a background of normal cells. In addition, multiple mutations present in a single sample were easily detected at the 1–5% level. An encoded series of human bronchoalveolar lavage samples was screened for *K-ras* mutations. Half of the samples assayed had at least one mutation, and these results were reproducible using at least two independent PCR reactions of each sample for LCR analysis. Although further clinical follow-up is required to determine the consequences of early *K-ras* mutations in the samples studied, the success with the BAL specimens suggests that the LCR assay will be a tool to study *K-ras* mutations in a variety of settings. The LCR results were confirmed by the independent methodology of cloning followed by mutant-specific oligonucleotide hybridization and confirmation sequencing.

Positive and negative controls were included in each set of LCR reactions to monitor both template-independent ligations and false ligations due to enzyme error. All PCR reactions were also assayed by LCR for presence of wild-type product to ensure that samples wild-type for the first base of *K-ras* codon 12 had successfully amplified the region in question. An encoded series of "unknowns" of *K-ras* codon 12 wild-type and mutant DNAs and negative control samples was correctly analyzed prior to work with the BAL specimens. Although

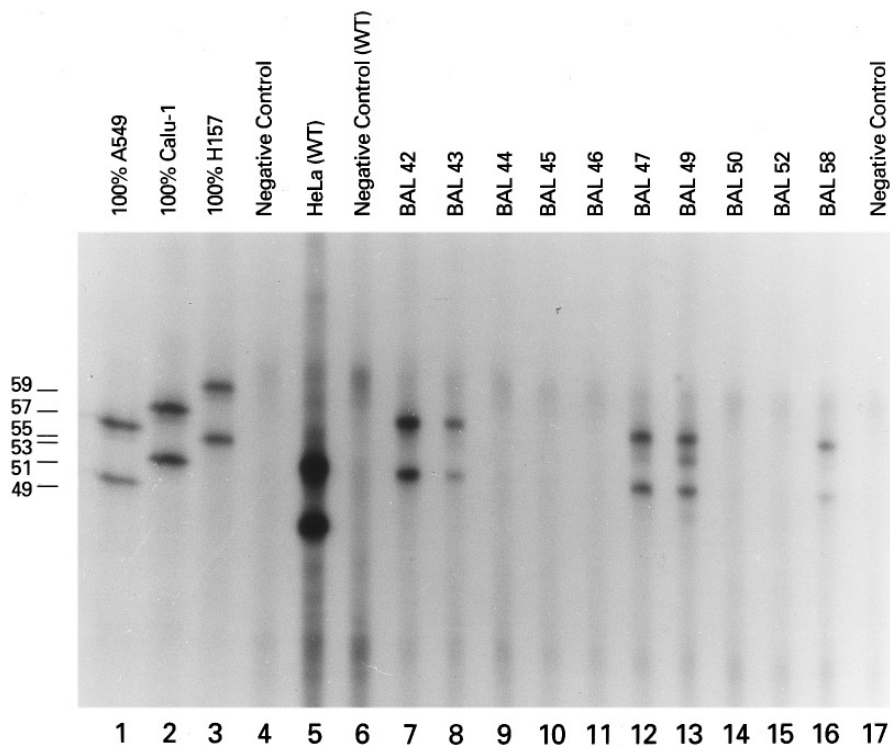


FIG. 4. PCR-based LCR analysis of BAL samples and positive and negative controls. DNA was isolated from a small fraction of each BAL sample, and PCR-based LCR was performed using the methods and controls described above. Mutant controls (lanes 1, 2, and 3) and wild-type control sample (lane 5) produced bands of the expected sizes. Of 10 BAL samples tested, 4 contained 1 mutation (GGT \rightarrow TGT) (lanes 7, 8, 12, and 16), 1 contained 2 mutations (GGT \rightarrow TGT and GGT \rightarrow AGT) (lane 13), and the rest were wild-type (lanes 9, 10, 11, 14, and 15). PCR negative controls analyzed by LCR with mutant primers (lanes 4 and 17) and wild-type primers (lane 6) were all negative.

further refinement of this assay is ongoing, the data produced using the current technique were independently verified in multiple replicate assays.

Other *K-ras* hotspot sites could be analyzed in the same LCR multiplex reaction by including specific primers of varying lengths which are complementary to the regions of interest. Potential targets include the second position of codon 12, as well as the first and second positions of codons 13 and 61. This is technically feasible, particularly with the successful adaptation of a nonisotopic methodology which is currently in progress.

Using the PCR-PIREMA technique, Jacobson and co-workers found *K-ras* mutations in 27 of 53 (51%) lung adenocarcinoma samples (15). This assay involves a series of PCR amplifications followed by DNA digestions which entail 75–115 PCR cycles to achieve a sensitivity of 0.1% detection of mutations (15). Although multiple controls were included to monitor cross-contamination, this technique requires extensive handling of PCR material, and utilizes a large number of PCR cycles. There is also additional cost and labor associated with the restriction enzyme digestion and analysis of fragments.

One practical advantage of the PCR-based LCR ap-

proach is that the number of amplification cycles is slightly less than with the PCR-PIREMA assay (65 cycles vs 75–115 cycles). The most important aspect may not be sensitivity if these two methods are comparable, but rather ease of assay and cost. The PCR-based LCR assay can be adapted to the plate assay format for rapid screening of a large number of samples (16) or automated DNA sequencer analysis using fluorescent primers (17). This procedure has potential for multiplex analysis of different possible mutational sites. A recent paper by Winn-Deen and co-workers detailed simultaneous analysis of 30 cystic fibrosis transmembrane conductance regulator mutations using fluorescent primers for multiplexed PCR and ligation analysis (17). This multiplexing allows several mutations to be determined in a single amplification, rather than from several individual reactions as is necessary for mutation-specific PCR methods. These advantages promise to bring point mutation detection to a more clinically relevant level. By combining the technique of a mutant enrichment (such as PCR-PIREMA) followed by LCR, additional sensitivity with fewer overall cycles of PCR may be feasible. Ongoing development is required to permit greater assay sensitivity while simultaneously reducing assay cost.

Recent data in colorectal cancer suggest that mutant K-ras may be present in apparently normal mucosa, and may be useful as a biomarker for colorectal cancer risk (18). Since K-ras mutations have been reported to occur early in adenocarcinoma of the lung (19), the additional sensitivity afforded by a combination enrichment-LCR technique may be quite important.

The evaluation of K-ras mutations for early lung cancer detection likely will require assays sensitive enough to detect informative cells at a lower density than in BAL fluid from a patient with primary lung cancer. Conversely, sensitivity should not be so high that K-ras mutations are reported which are of uncertain clinical relevance. BAL fluid from a high-risk group such as the previously resected patients of this study may be more representative of the condition of the bronchial epithelium in a person with a developing lung cancer. Further research is needed to determine the K-ras mutation detection sensitivity required to permit clinically meaningful early lung cancer detection.

With the use of a highly sensitive assay to detect K-ras mutations, certain sampling problems must be discussed. The number of informative bronchial epithelial cells recovered from the airway during a bronchial lavage is variable. A clonal population of K-ras-positive cells may exist in the airway but the lavage may have failed to capture any of the exfoliated cells. The heterogeneity of cell populations within a bronchial lavage specimen may result in different K-ras mutation patterns within different portions of the same specimen. In the early stages of consideration of molecular diagnostics for clinical application, well-designed trials must be conducted to evaluate these sampling problems. The ultimate benefit and acceptance of new diagnostic tools will be improved if clinicians are appraised of both strengths and weaknesses of these assays.

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