A rapid PCR ELISA for the detection of activated K-ras in colorectal cancer

R L Ward, F Santiago, N J Hawkins, D Coomber, T O'Connor, A V Todd

Abstract

Aims—To develop a rapid PCR ELISA procedure for the detection of mutations in K-ras in a microtitre plate format, and to evaluate the assay for the detection of these mutations in human colorectal cancer.

Methods—An enriched PCR method was used with labelled primers, and PCR product was captured on GCN4 coated immunoassay plates. Detection of biotinylated mutant product was performed by colorimetric assay with streptavidin-horseradish peroxidase. The assay was used to determine K-ras status in a series of 60 human colorectal neoplasms, together with paired normal colonic mucosa. Results from gel electrophoretic analysis were compared with ELISA results.

Results—The assay proved reliable in detecting K-ras mutations in DNA extracted from both fresh and paraffin embedded colorectal tumours. ELISA results were comparable with results from gel electrophoresis. Mutations of K-ras were detected in 16 of 48 adenocarcinomas and five of 12 adenomas but no mutations were detected in normal mucosa. There was a highly significant difference ($p < 0.0005$) between optical density values for carcinomas with mutant K-ras and their paired normal data. Adenomas did not show the clear distinction between positive and negative results seen with carcinomas.

Conclusions—This assay provides a rapid and reliable means of detecting mutations in codon 12 of the K-ras oncogene. The single tube format colorimetric analysis in microtitre plates and clear discrimination between mutant and wild type genes makes the assay suitable for automation. The occurrence of intermediate results in the case of adenomas provides support for the hypothesis that mutations of K-ras occur early in the course of colorectal carcinogenesis.

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Keywords: K-ras, colorectal neoplasia, point mutation, ELISA, PCR.

Over the last eight years, many studies have confirmed the initial reports of frequent point mutation of the K-ras proto-oncogene in human colorectal neoplasms.¹⁻³ It has been postulated that these mutations occur early in the course of colorectal carcinogenesis⁴ and that their detection may allow diagnosis of tumours at a stage where curative surgery is

possible.⁵ Over the next few years, the likely development of treatments targeting the mutant K-ras product will provide even further impetus for the development of rapid and simple detection strategies.

Although there have been many published protocols for the detection of point mutations in the ras oncogene family, these protocols differ widely in their sensitivity and complexity.³ Typically these assays rely on polymerase chain reaction (PCR) amplification of the K-ras gene, with subsequent analysis of the product by a variety of methods. Early protocols allowed the detection of mutant alleles in 1-10% of cells.³ More recently, assays of greater sensitivity have been developed, including allele specific amplification,⁶⁻⁸ induced restriction fragment length polymorphisms plus hybridisation,⁹⁻¹² cloning plus hybridisation,¹³ enriched PCR,^{14,15} and enriched PCR plus allelic specific oligonucleotide hybridisation.^{12,14}

Assays that will be of use in a routine diagnostic laboratory will be not only be sensitive but robust, rapid, and specific. We have developed a new protocol for the detection of point mutations in codon 12 of K-ras which is suitable for automation. The protocol allows the selective amplification of mutant K-ras oncogenes from tumour tissue through the creation of unique restriction enzyme sites. A colorimetric plate assay is then used for the rapid analysis of mutant product. In this report, we describe the evaluation of the assay on a series of human colorectal neoplasms.

Methods

TISSUE AND DNA PREPARATION

After obtaining informed consent, 59 consecutive individuals undergoing surgical resection of either adenomas or adenocarcinomas of the colon or rectum at St Vincent's Hospital, Sydney, were enrolled in this prospective study. Fresh representative samples (500 µg) of the tumour and a paired sample of normal mucosal tissue proximal to the tumour were immediately frozen at -70°C . In addition, the assay was performed on paraffin embedded blocks of the same tumour tissues, and from normal tissues from the proximal resection margin. The paraffin embedded tissues were obtained from the Department of Anatomical Pathology, St Vincent's Hospital, following routine processing. In each case the histopathological diagnosis, Dukes' stage, and tumour size were determined independently by a histopathologist within the Department of Anatomical Pathology, St Vincent's Hospital.

Forty eight adenocarcinomas were assayed

St Vincent's Hospital,
Darlinghurst,
NSW 2010, Australia:
Department of
Medical Oncology
R L Ward
N J Hawkins
D Coomber

Department of
Colorectal Surgery
T O'Connor

The R W Johnson
Pharmaceutical
Research Institute,
Sydney, Australia
F Santiago
A V Todd

Correspondence to:
Dr R L Ward.

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Figure 1 Schematic diagram of the single tube PCR-ELISA. First round amplification uses the untagged 3KiE primer, and the biotinylated primer 5BKIM, which contains a strategically placed mismatch which induces a BstNI restriction site if codon 12 is not mutated. Following amplification, BstNI digests homoduplex wild-type product, thus providing a mutant enriched template second round amplification. The primer 3AKIL contains a 12 base recognition sequence for the DNA binding protein GCN4, thus permitting capture on GCN4 coated microtitre plates. Homoduplex wild-type molecules are cleaved before colorimetric assay, but mutant homoduplex molecules and heteroduplexes molecules have sequences for both capture and detection with streptavidin-horseradish peroxidase (SA-HRP).

from 48 individuals (22 male, 26 female) ranging in age from 31 to 91 years, with an average age of 69.0 (SD 12.0) years. Two of these tumours were Dukes' stage A,¹⁶ while 17 were stage B, 28 were stage C, and one was stage D.¹⁷ Likewise, 12 adenomas were assayed from 11 individuals (seven male, four female) ranging in age from 56 to 83 years, with an average age of 72.4 (7.1) years. Paired samples were obtained from all patients, including two from each of four patients who had two synchronous tumours.

DNA was extracted from the frozen tissues by first mincing them in a dry ice/methanol bath, then incubating the samples overnight at 48°C in a solution of 1% sodium dodecyl sulphate (SDS), 500 µg/ml proteinase K in 500 mM Tris, 20 mM ethylenediamine tetraacetic acid (EDTA), 10 mM NaCl, pH 9.0.¹⁸ Following phenol/chloroform extraction and ethanol precipitation, the DNA was re-suspended in 10 mM Tris HCl plus 1 mM EDTA, pH 8.0, and the DNA concentration was estimated by spectrophotometry. For the

preparation of DNA from paraffin embedded tissues, 4 µm sections were cut from the blocks. After each block was cut the blade was washed with xylene in order to prevent cross contamination. Four sections were immersed in 400 µl of extraction buffer (10 mM Tris HCl, pH 8.3, 50 mM KCl, 2.5 mM MgCl₂, 0.45% Tween-20, and 0.5 mg/ml proteinase K),¹⁵ incubated overnight at 55°C, and then boiled for 10 min. A 10 µl aliquot of the aqueous phase was used as the template in subsequent PCR reactions. In each instance, an adjacent section was stained with haematoxylin and eosin, and examined by light microscopy to confirm its histological appearance. DNA extracted from the colonic cancer cell line SW480, known to be homozygous mutant at codon 12 of the K-ras oncogene,¹⁹ was used as a positive control, while DNA from the homozygous wild type cell line K562 was used as a negative control.

MUTATION ANALYSIS

Mutations at the first and second bases of codon 12 of K-ras were detected using single tube allele specific enriched PCR followed by an ELISA assay. This protocol is represented schematically in fig 1 and the sequence of the primers is shown in table 1. Analysis was performed by one of the authors (FS), who was masked to respect to the tissue of origin of each sample. Each PCR was performed at least twice, and the reaction products were assayed in triplicate on each occasion.

The first round of PCR amplification was performed using 0.5 µg of DNA extracted from fresh tissue or 10 µl of supernatant extracted from paraffin embedded tissues. Reaction mixtures also contained 2.5 mM Mg²⁺, 100 µM deoxynucleotide triphosphate (dNTP), 5 pmol of each primer (5BKIM and 3KiE), and 1.0 unit Taq DNA polymerase in PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3) to a total volume of 50 µl. The reactions were cycled in a Perkin-Elmer 9600 PCR cyclor as follows: 1 × 94°C for 4 min, 25 × (58°C 20 s; 74°C 20 s; 94°C 20 s), 1 × 99°C for 30 min, and 25°C for 5 min. BstNI (40 units in 4 µl; New England Biolabs) was added, and the tube incubated at 60°C for 90 min, then 99°C for 10 min.

The second round of amplification was performed after spiking additional reagents into the reaction tube including the nested primer 3AKIL and other reagents to give a final reaction mixture of 1.25 mM Mg²⁺, 100 µM dNTP, 100 pmol of each primer (5BKIM and 3AKIL), and 1.0 units Taq DNA polymerase in PCR buffer to a volume of 100 µl. The reaction was then cycled as follows: 25 × (65°C 20 s; 74°C 20 s; 94°C 20 s), and 1 × 99°C for 30 min. An aliquot of this second reaction was then removed for overnight digestion with BstNI (1 U/µl in a buffer of 10 mM Tris HCl, 10 mM MgCl₂, 50 mM NaCl, 1 mM 1,4-dithiothreitol, pH 7.9, and bovine serum albumin 100 µg/ml). The samples were analysed by electrophoresis on a 5% Nu-sieve agarose gel and by enzyme linked immunosorbent assay (ELISA) plate analysis.

Table 1 Oligonucleotides for the detection of K-ras mutations at codon 12

Name	Oligonucleotide sequence
3KiE	5'CTCATGAAAATGGTCAGAGAAAC3'
5BKIM	5'*GACTGAATATAAACTTGTGGTAGTTGGA ^c CT3'
3AKIL	5'GGATGACTCATTTTCGTCCACAAAATGATTCTGAATTAG3'

* Denotes biotinylation; italicised sequence is the tag which contains the GCN4 recognition site; lower case base in the 5BKIM primer indicates mismatch with K-ras gene.

Figure 2 Identification of K-ras using single tube enriched PCR. DNA was extracted from the homozygous wild type cell line K562 (–), and from six colorectal carcinomas (T1–6) and corresponding normal mucosa (N1–6). Products are shown on a 5% NuSieve gel stained with ethidium bromide. MW=phiX174/HaeIII. Mutant K-ras (103 bp band) is present in two tumours. Median optical density values from ELISA analysis of each product are shown, with strongly positive values corresponding to mutant product.

Colorimetric assay of reaction products was performed using the Captagene-GCN4 detection kit (Amrad) according to the manu-

facturer's instructions. Briefly, wells of a microtitre plate, which had been precoated with GCN4, were blocked with 10% non-fat milk in phosphate buffered saline (PBS) for 10 min at room temperature. An aliquot of digested product, equivalent to 7 µl of the final PCR reaction, was added to triplicate wells in PBS/0.05% Tween-20 containing 10% non-fat milk and 4 µg/ml sonicated double stranded salmon sperm DNA, and incubated for 30 min at room temperature. Following four washes in PBS/0.05% Tween-20, streptavidin-horseradish peroxidase conjugate was added for 30 min at room temperature. After a further six washes, 100 µl of substrate (93 mM sodium acetate, 1 mM citric acid, 0.127% H₂O₂ [6% w/v], and 0.1 mg/ml tetramethyl-benzidine) were added to each well, and after colour development the reaction was terminated with 100 µl of 0.5 M H₂SO₄. The absorbance was determined by Dynatech MR5000 plate reader using a 450 nm test filter and a 630 nm reference filter. Positive and negative controls for the kit were including for all assays.

STATISTICAL ANALYSIS

In the ELISA reaction, all PCR products were assayed in triplicate, and the median value for each sample was used in subsequent analysis. Comparison between the value obtained from normal mucosa and its corresponding adenoma or adenocarcinoma was performed using a paired *t* test. Results were considered significant at *p*<0.01, based on the Bonferroni adjustment for multiple comparisons.²⁰ Normal samples were not available for assessment in the case of three patients, from whom four tumour samples had been obtained. In these cases, the corresponding tumour specimens were omitted from the paired analysis.

The relationship between the presence of mutations in K-ras and pathological variables such as Dukes' stage of carcinomas and size and histological type of the adenomas was investigated using logistic regression analysis.

Results

Under the conditions described above, the single tube enrichment procedure was able to identify mutant K-ras genes in DNA from the homozygous mutant cell line SW480, as evidenced by a single undigested 103 bp product. This product was not observed in the homozygous wild type cell line K562 (fig 2), where 73 bp and 30 bp fragments of the digested wild type product were observed. The limits of detection of this assay were determined by serial dilution of SW480 DNA in K562 DNA at ratios from 1:10 to 1:10 000. A ratio of 1 mutant gene to 1000 wild type genes was sufficient to give a signal significantly greater than that seen with wild type genes alone (data not shown).

Before analysis by ELISA, the presence of either mutant or wild-type K-ras PCR products in all 116 frozen tissue samples was assessed by gel electrophoresis. All samples contained 73 bp and 30 bp products, while some of the tumours contained an additional band at

Figure 3 Frequency distribution of fresh frozen samples analysed using single tube PCR ELISA. Samples were categorised according to the median of triplicate optical density (OD) values in one assay (x axis). Numbers of samples in each OD range are shown as a percentage of all samples for each group of either normal mucosal samples, adenocarcinomas, or adenomas (y axis). Numbers of specimens in each group are shown.

Table 2 Number of adenomas showing mutant K-ras, grouped according to histological diagnosis and size

	<1 cm		1-2 cm		>2 cm		Total
	Mutant	Wild	Mutant	Wild	Mutant	Wild	
Tubular	0	2	0	0	0	0	2
Tubulovillous	0	2	2	0	2	2	8
Villous	0	0	0	0	1	1	2
Total	4		2		6		

103 bp, indicating the presence of mutant K-ras (fig 2). The results seen on gel electrophoresis were comparable to those seen using the ELISA assay (fig 2), where only samples containing a 103 bp product gave a strongly positive signal.

The frequency distribution of ELISA results from these samples is shown in fig 3. The values obtained from adenocarcinoma specimens fell into two distinct groups. The test negative group had a mean optical density (OD) of 0.10 (SD 0.04), while the test positive group had a mean OD of 1.183 (0.19). The values for normal mucosal samples from these patients with carcinoma had a mean OD of 0.08 (0.04). An arbitrary point of OD 0.5 was chosen to distinguish between these groups, and all samples were thus characterised as either containing mutant K-ras (test positive group) or not containing mutant K-ras (test negative group). On the basis, there was a significant difference in OD readings between carcinomas with mutant K-ras and their paired normal mucosa (paired *t* test, $p < 0.0005$). However, no such difference was observed between carcinomas without K-ras mutations and their paired normal mucosa ($p = 0.06$). No normal mucosal samples contained mutant K-ras at codon 12. However, 16 of 48 carcinoma samples (33%) did contain this mutation. Using logistic regression analysis, we were unable to demonstrate any significant association between the presence of mutations in K-ras and the Dukes' stage of the carcinoma.

In contrast to the adenocarcinoma population, a clear distinction between high and low values was not apparent among the adenomas examined in this study (fig 3). Six of the 12 adenomas examined showed low OD values, while with an arbitrary cut off of OD 0.5, five of the 12 adenomas (42%) would be classified as containing mutant K-ras. While there was a tendency towards an increase in mutant K-ras in the larger adenomas and those with at least some villous component (table 2), this did

not reach statistical significance in the small population analysed. In the four cases where an adenoma and carcinoma were collected from the same individual, two of the four carcinomas contained mutant K-ras, while all four of the adenomas contained only wild-type K-ras.

DNA was extracted from paraffin sections of tumours known to be either mutant ($n = 5$) or wild ($n = 5$) for K-ras, together with sections from paired normal mucosa. For all samples, analysis using the single tube PCR ELISA gave results concordant with those obtained from fresh frozen specimens (data not shown).

To examine further the potential value of this assay in the diagnosis of colorectal carcinomas, its sensitivity and specificity for predicting disease status was determined at a variety of cut off points, as shown in table 3.

Discussion

Several enriched PCR methods have previously been described for the detection of mutant *ras* genes. We have sought to modify these protocols in order to make them more amenable to automation. Firstly, we have streamlined the protocol, so that two rounds of amplification and the intermediate digestion of the K-ras product can be performed in a single tube. This limits the need for the manipulation of reaction product, thereby reducing both the time and cost of the reaction and the likelihood of false positive results arising from carryover of PCR product. Secondly, the incorporation of biotin and GCN4 labels in the primers allows colorimetric detection of PCR products. While this does not increase the sensitivity of detection significantly in comparison to Nu-sieve gel electrophoresis, it does remove observer bias in the interpretation of assay results. Furthermore, the use of a microtitre plate format allows the rapid and simultaneous analysis of a large number of samples, thereby offering the potential for further reduction of the time and cost of analysis through automation.

Importantly, our assay also provides an in-built positive control for template amplification, through colorimetric analysis of an aliquot of the PCR reaction which has not been subject to final digestion with *Bst* NI. This is because an undigested sample will always generate a positive signal provided that amplification of the template, either wild or mutant, has occurred. Such an inbuilt control is important in distinguishing between reactions which are negative because of complete digestion of wild-type product, and those in which amplification of a PCR product has failed to occur.

These significant improvements in throughput and analysis have been gained without loss of sensitivity, as the protocol allows the detection of less than one mutant gene in a background of 1000 wild type genes, a degree of sensitivity similar to that already reported.¹⁵

We determined the frequency of mutations at codon 12 of K-ras in an Australian population to be 33% for carcinomas and 42% for adenomas. Clearly, the use of an assay which also detects mutations at codons 13 and 61 is

Table 3 Sensitivity and specificity of the single tube PCR ELISA for determining the presence of colorectal carcinoma in a test population of 48 colorectal carcinoma samples and 48 matched normal mucosa samples at a variety of arbitrary cutoff points

Cutoff OD	Sensitivity	Specificity
0.08	0.77	0.73
0.10	0.60	0.91
0.11	0.48	0.96
0.15	0.40	0.98
0.2	0.35	0.98
0.3	0.33	1.00
0.5	0.33	1.00
0.8	0.33	1.00
1.0	0.27	1.00

OD = optical density.

likely to have resulted in an increase in the frequency of detection of K-ras mutations, perhaps of the order of 5–10%.^{4,21} Likewise, any heterogeneity of K-ras mutations within the tumour may result in an underestimation of the true incidence of mutations in the tissues examined, although such heterogeneity has not been found in colorectal neoplasms. Nevertheless, our findings are in broad agreement with observations made from other populations using different assay techniques.^{1,22} The frequency of K-ras mutations in adenomas has been shown to vary with size and histological classification,^{4,22,23} although the relationship to size has not been reported by workers.²⁴ Although such trends were noted in this study (table 2), we were unable to show any significant associations because of the small numbers of adenomas assessed.

The assay was able to make a clear distinction between those carcinomas with normal K-ras and those with mutations of the oncogene at codon 12, an outcome consistent with the design of the test, which seeks to specifically enrich for K-ras mutations where those are present. While the results obtained from adenoma samples tended to cluster in the normal and mutant groups seen with carcinomas, two specimens showed results at an intermediate level (OD 0.55–0.60). It may be possible that these findings reflect amplification from a very small fraction of mutant cells within the tumours. If this is so, then such a small population of mutant cells may occur much less commonly in carcinomas, as we found no results of an intermediate nature in this group. Such an observation is consistent with reports that mutations of K-ras occur early in the course of colorectal carcinogenesis.⁴

It is noteworthy that no mutant K-ras was detected in 56 samples of normal mucosa. It has been reported that mutations of K-ras are present in seemingly normal mucosa from individuals with K-ras positive neoplasms,²⁵ perhaps secondary to a field effect induced by unspecified carcinogens. The results in this study argue clearly against this possibility, at least in this Australian population.

While we have described this assay with reference to frozen and paraffin embedded tissues, its characteristics make it potentially useful for the automated screening of large numbers of samples, in which small numbers of mutant cells are present. One such example would be in the screening of faeces from patients known to be at risk of colorectal cancer.

When used to analyse frozen sections, our assay showed a high degree of specificity for colorectal cancer at cutoff points above OD 0.3 (table 3). However, its sensitivity was clearly limited by the frequency of K-ras mutations in the tumours studied. Improvements in sensitivity of the assay may potentially be achieved by the analysis of additional oncogenic mutations. While these conclusions can only be justified if the results from frozen tissues are reproducible on faecal samples, it is encouraging that in preliminary studies we have been able to detect K-ras in faeces from patients with colorectal cancer. It is hoped that such strat-

egies for the detection of oncogene mutations in colorectal neoplasms may one day provide a rational basis for therapeutic intervention.

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