Relative expression of wild-type and activated Ki-*ras*2 oncogene in colorectal carcinomas

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Abstract. A quantitative, competitive RT-PCR-RFLP assay was developed to detect and discriminate the expression of mutant versus wild-type alleles of the Ki-*ras* oncogene. The aim was to establish whether these alleles are differentially expressed in human malignant neoplasma, since experiments *in vitro* have shown stoichiometric representation and expression of *ras* genes does not necessarily engender a cancer phenotype. Sixteen primary colorectal carcinomas and two colorectal carcinoma xenografts, passed in immune-suppressed mice, were studied. Previous sequence analysis had established that 9 of the primary tumours and both xenografts had codon 12 Ki-*ras* mutations, 4 tumours had codon 13 mutations and 3 were wild-type controls. Wild-type and mutant Ki-*ras* were co-expressed in all the primary tumours, but the assay showed that stoichiometrically equivalent amounts of the two mRNA species were present in only one-third: in the others, mutant Ki-*ras* was overexpressed by around 30-60% relative to wild-type. The xenografts showed a similar range of values, despite their near-total lack of stroma. Ki-*ras* activation by point mutation is known to be involved in the early, adenoma phase of evolution of colorectal tumorigenesis, but these results show that differential expression of the mutant allele is common in carcinomas and may be associated with persisting growth advantage.

Introduction

Genetic alterations are known to be responsible for cancer. The initiation, progression and promotion of tumours has been associated with a sequence of such genetic aberrations involving oncogenes and oncosuppressor genes (1,2). In human colorectal cancer several of these critically altered genes are known, including APC, MCC, Ki-*ras*, DCC and p53 (3,4). The alterations in the function of these genes may be responsible for specific stages of tumorigenesis and cancer progression (3). Activation of Ki-*ras* by point mutations at codons 12, 13 and 61 appears in the premalignant phase of colorectal tumorigenesis, being observed in the larger (and more dysplastic) adenomas. Carcinomas show the same spectrum of mutations, at approximately the same incidence, around 40-50%.

In a variety of experimental systems, expression of activated *ras* genes is associated with early stages of carcinogenesis, being apparently responsible for both initiation and progression (1,2,5-8). Recent work, however, has emphasised the importance of gene dosage in the phenotype of cells bearing *ras* oncogenes (9-11). Whilst the presence of mutated and wild-type *ras* genes in stoichiometrically equivalent amounts may have little effect on phenotype (12), chemically-induced carcinomas and papillomas of skin frequently differ in that carcinomas tend to have more gene copies of mutated than wild-type *ras* (6,9,10).

In this study we sought to characterize more fully the role of Ki-*ras* in human colorectal cancer. In view of reports that *ras* p21 is less abundant in advanced than in early tumours (22), we asked whether expression of the abnormal Ki-*ras* oncogene makes a continuing contribution to the development of malignant tumours. We also asked whether wild-type and mutated genes are equally expressed. To do this we developed a competitive RT-PCR-RFLP assay (13,14) using an exon connection strategy in which an antisense primer within exon 3 was coupled with the sense primer in exon 1 already described (15). The exon 3 primer also bore a nucleotide mismatch that created a BstNI site at the 3' end of the PCR product. This controlled for digestion efficiency at a second BstNI site at the 5' end of the product, which was destroyed by mutation in codon 12 (Fig. 1).

Using this technique we examined the ratio of expression of wild-type and mutant Ki-*ras* genes in primary colorectal cancers in man. The results show that mutant Ki-*ras*, when present, is always expressed, and its expression often exceeds that of wild-type allele.

Materials and methods

Tumour material. Tumour fragments from colorectal carcinomas were snap frozen in liquid nitrogen after surgery

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and stored at -70°C. All tissues were carefully trimmed to remove as much non-neoplastic tissue as possible. A total of 16 colorectal carcinomas were examined, 9 with known codon 12 Ki-*ras* mutations, 4 with codon 13 mutations and 3 without mutation. To control for the effect of non-neoplastic stromal cells on the analysis, two further carcinomas were studied following multiple passage as xenografts in immune-suppressed mice. Although such xenografts faithfully reproduce the histology and genetic defects of the primary tumours (16), they possess little non-neoplastic stroma, which in any case is of murine origin and so affords an inefficient template for the human-specific PCR primers.

Cell lines. Two human colon carcinoma cell lines Colo 320 and SW 480 were employed for quantitative determination of the ratio between normal versus mutant Ki-*ras* transcripts: Colo 320 cells express only wild-type Ki-*ras*, whilst SW 480 express only Ki-*ras* with a codon 12 mutation.

RNA extraction. RNA was extracted by the RNazol B kit (BioGenesis) which is a modified version of the guanidinium isothiocyanate method. In brief, approximately 10 mg of frozen tissue were homogenized in 800 µl RNazol B. After addition of 100 µl chloroform the mixture was shaken vigorously and centrifuged for 20 mins at 12000g at 4°C. The supernatant was collected and the RNA precipitated with an equal volume of isopropanol. The RNA pellet was washed sequentially with 4M LiCl and 75% ethanol.

The quality and quantity of RNA was assessed by formaldehyde denaturing agarose gel electrophoresis and spectrophotometry at 260 nm and 280 nm.

cDNA synthesis. 1 µg of total RNA was primed with 0.5 µg oligo-dT and the reaction was performed in a final volume of 20 µl with 200 U MMLV reverse transcriptase (BRL) following the supplier's protocol. After 1 hour at 37°C the enzyme was heat inactivated (5 min, 98°C). A negative control without RNA was also included.

PCR amplification. The cDNA was amplified in a 100 µl reaction (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% Triton, 200 µM dNTPs) with 200 ng of each primer and 2.5 U Taq polymerase (NBL). The amplimer sequences were
5'-ACTGAATATAAACTTGTGGTAGTTGGACCT-3' and
5'-CAAATCACATTTATTTCCTACCAGGACCAT-3'.
After an extended initial denaturation step (94°C for 4 min) the PCR program consisted of 1 min at 94°C, 1.30 min at 62°C and 1.30 min at 72°C for a total of 25 cycles.

To exclude false positive results due to Ki-*ras*1 pseudogene in DNA still present in the RNA preparations, control RNA was subjected to PCR amplification without prior incubation with reverse transcriptase.

PCR amplification for detection of LOH at genomic DNA level was performed as described (17,18).

RFLP analysis. For codon 12 mutations 20 µl of the PCR reaction were subjected to BstNI (NEB) digestion (20 U) at a final volume of 30 µl at 60°C for 3 h. For codon 13 mutations the same procedure was employed as for codon 12 except

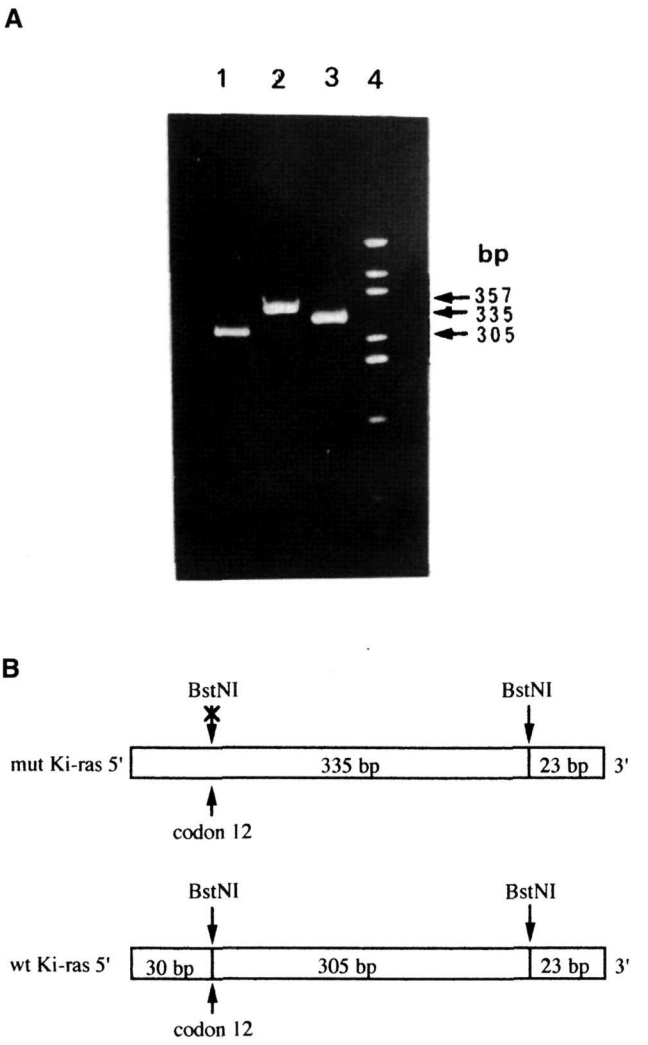

Figure 1. (A) Application of competitive RT-PCR-RFLP on Colo 320 RNA reveals only normal Ki-*ras* signal being expressed while in SW 480 only codon 12 mutant Ki-*ras* signal is present. Lane 1: Colo 320, lane 2: undigested PCR product, lane 3: SW 480, lane 4: marker. (B) Nucleotide substitution in the primers facilitate the creation of two BstNI sites. The 3' end site is permanently functional and serves as a control for the efficiency of digestion, discriminating RFLP products from undigested PCR products. The 5' end site is functional depending upon the presence of codon 12 mutations.

HphI (NEB) was used at 37°C. The products were electrophoresed through a 3% agarose gel (3 parts Nusieve/1 part Seakem). Gels were stained with ethidium bromide and photographed on an ultraviolet light transilluminator.

RFLP analysis for detection of LOH was performed as described (17,18).

Ratio quantitation. Total RNA from Colo 320 and SW 480 cell lines was mixed in the following mass ratios to create 5 batches of mixtures: 1/1, 0.5/1, 0.25/1, 0.125/1, 0.0625/1 (Colo 320/SW 480). 3 µg of each mixture was used for the RT-PCR-RFLP assay.

The RFLP products were loaded on 6% mini polyacrylamide gels containing 1% glycerol. Gels were run in TBE buffer at 100V for 1 h. After electrophoresis, gels were stained in ethidium bromide and photographed on a UV

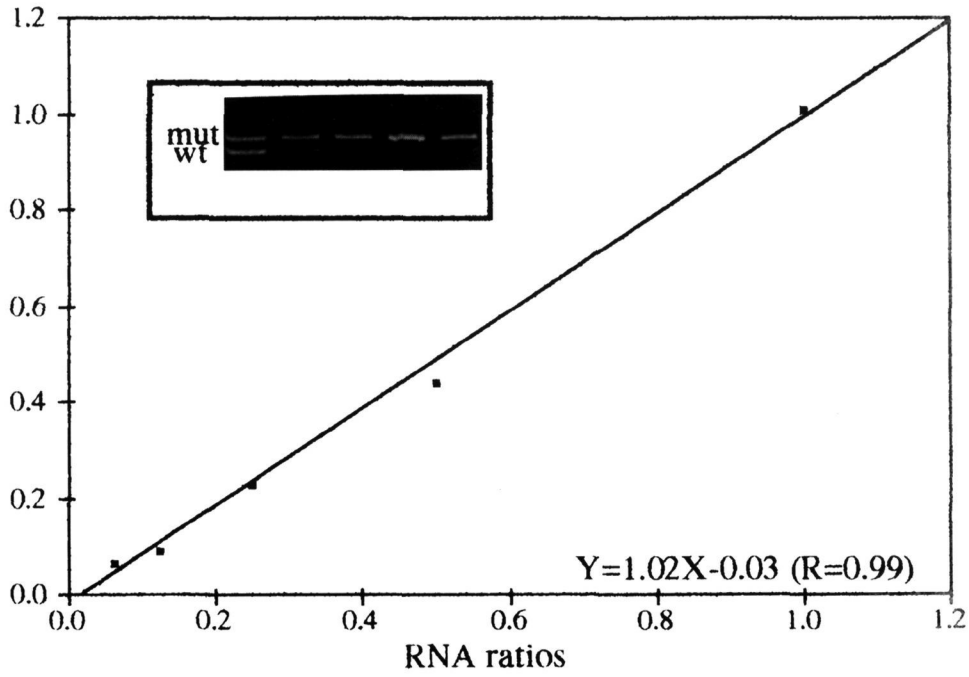

Figure 2. Regression analysis between total RNA ratios of normal versus mutant Ki-*ras* resulted in a linear relationship described by the equation Y=1.02X-0.03 with a coefficient of correlation R=0.99.

transilluminator. The photographs were scanned on a flatbed scanner (Microteck) and computer image analysis was performed on a Macintosh computer. The densitometric values from computer image analysis were statistically analysed with the aid of Statworks (1.2) on the same computer.

The RFLP products from tumour samples were analysed in the same way. Densitometric values for each tumour taken from two independent experiments, and the average values were used to compute the ratio between the mutant and wild-type Ki-*ras* transcripts, using the cell line data as a calibration curve.

Results

Ratio quantitation. To obtain reliable measurements for the ratio of the level of expression between the trasncripts of the Ki-*ras* locus, we assessed the accuracy of the competitive RT-PCR-RFLP assay, using RNA extracted from the two human colon carcinoma cell lines Colo 320 (wild-type only) and SW 480 (codon 12 mutant only) (Fig. 1). Total RNA from these cell lines was mixed in various ratios, as described in the Methods and competitive RT-PCR-RFLP was performed with various PCR cycles. The ratios of Ki-*ras* obtained after the RFLP analysis was plotted against the ratios of the initial total RNA from the two cell lines, giving a standard curve (Fig. 2). Under optimum conditions (25 cycles) the data points closely fit a linear equation (R=0.99). The equation describing this standard curve is Y=1.02X-0.03, indicating a direct linear proportionality between the input RNAs and the cDNAs amplified from the transcripts. Higher cycle numbers were avoided, however, as this linear relationship was lost, presumably because of the accumulation of heteroduplex products (13).

Codon 12 mutation analysis. All 9 carcinomas with codon 12 mutation expressed both mutated and wild-type Ki-*ras* transcripts (Fig. 3A). Densitometric analysis of the samples showed that the ratio of mutated and wild-type transcripts was close to unity (0.8-1.0) in only 3 (Table I). In all others, mutant Ki-*ras* was overexpressed relative to wild-type by 30-60%. All these ratios of wild-type to mutant Ki-*ras* cDNA observed in the carcinomas fall within the range shown to be linearly related to transcript in Fig. 2.

Codon 13 mutation analysis. All 4 cases with codon 13 mutation had the same Asp 13 mutation in the genomic DNA. This mutation creates a recognition site for the HphI restriction enzyme which is unique in the amplified cDNA fragment. Hence, cDNA mutant from transcripts is digested with this enzyme while wild-type cDNA is not affected.

Analysis of the 4 carcinomas with Asp13 mutation revealed that both mutant and wild-type transcripts were expressed in all (Fig. 3C). Because of the lack of a second restriction site in the PCR product, the efficiency of digestion with the HphI cannot be controlled. For this reason we did not attempt to quantify the ratio between the Ki-*ras* transcripts, but qualitatively it was clear that, as with codon 12 mutations, both mutant and wild-type genes were expressed.

Control tumour tissues. Analysis of the expression of codon 12 mutant Ki-*ras* in 2 xenografts showed values that covered the same range as the primary tumours: both mutant and wild-type alleles were expressed in both xenografts, one in near stoichiometric equivalence, the other with the mutant in excess by around 70% (Fig. 3B and Table I). As expected, the three carcinomas without Ki-*ras* mutation showed only wild-type cDNA (Fig. 3A tracks 2,3,7).

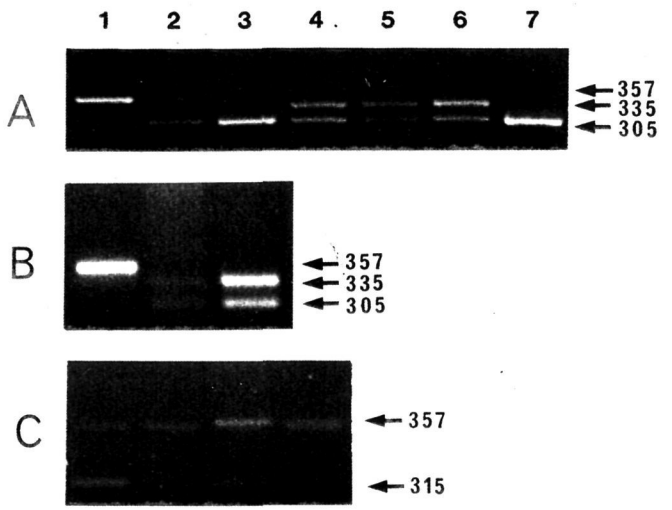

Figure 3. Results from the RFLP analysis. (A) codon 12 mutation analysis, lane1: undigested product, lanes 2, 3, 7: products from colon carcinomas with no codon 12 mutation after RFLP analysis, lanes 4, 5, 6: heterozygous products after RFLP analysis, (B) codon 12 xenograft analysis, lane 1: undigested product, lanes 2, 3: heterozygous mRNA Ki-*ras* products from xenografts after RFLP analysis, (C) codon 13 mutation detection in four heterozygous tumours.

Table I. Ki-*ras* codon 12 mutations detected by competitive RT-PCR-RFLP analysis in colon tumours and RFLP data from polymorphic sites close to or within Ki-*ras* locus.

Samples[a]	Mutation[b]	RNA ratio (wt/mut)	RsaI	StuI	BstXI
1	ASP12	0.66		A2	A1A2
2	SER12	0.95		A2	A1A2
3	ASP12	0.61	A1	A1A2	
4	ASP12	0.77	A1	A1	A1
5	ALA12	0.86	A1	A1A2	
6	VAL12	0.88			
7	VAL12	0.68			
8	ASP12	0.57		A1	A1A2
9	ASP12	0.61	A1	A2	A2
Xen 1	VAL12	0.98			
Xen 2	VAL12	0.59			

[a]Xen=Xenograft tumours; [b]codon 12 amino acid substitutions; A1=allele 1; A2=allele 2.

RFLP analysis for loss of heterozygosity at the Ki-ras locus. Five of the 9 patients with carcinomas bearing codon 12 mutations were informative at polymorphic sites close to or within Ki-*ras*2 (Table I). In every case, including 3 carcinomas in which the ratio of wild-type to mutant transcript was less than 0.67, both alleles were clearly present within the tumour, apparently in approximately equal amounts. Thus there was no evidence for loss of heterozygosity at the Ki-*ras* locus to account for the differences in expression between the Ki-*ras* alleles described above.

Discussion

We have described a quantitative RT-PCR method capable of discriminating mutant from wild-type Ki-*ras* transcripts in human tumour tissues. In this method, the ratio of the two species of transcript is a direct linear function of the ratio of their amplified, reverse-transcribed cDNAs. We attribute the simplicity of this relationship to the fact that no correction is required to match the efficiencies of synthesis of the mutant and wild-type products: during both reverse transcription and the polymerase chain reaction, synthesis of both proceeds within the same reaction vessel from the same primers, and PCR products are of identical length. Initially we were concerned that the relationship between RNA transcript and amplified cDNA might be significantly distorted by the extent of heteroduplex formation, since this could influence the kinetics of denaturation and reannealing. This may have been minimised by the relatively low number of cycles used.

The results show that mutant Ki-*ras*, if present in colorectal carcinoma DNA, is invariably expressed. Expression of wild-type Ki-*ras* never significantly exceeded that of the mutant, but often was substantially less. Loss of heterozygosity at the Ki-*ras* locus is unlikely to be the explanation for this preferential expression of the mutated Ki-*ras* allele, since a wild-type Ki-*ras* allele was present together with the mutant allele, in every case where this could be ascertained. Moreover, LOH involving chromosome 12p is one of the rarest defects documented in allelotypes of large series of human colorectal carcinomas (19), although selective loss of wild-type Ki-*ras* has been documented in a small number of cases (20). The data from the cases presented here therefore point to the conclusion that mutated Ki-*ras* genes, when present in colorectal carcinoma cells, tend to be expressed more than the wild-type genes by virtue of a transcriptional or post-transcriptional mechanism.

Quantitative analysis of data gathered from authentic tumour tissue, as here, is often uncertain because of the unknown extent to which intra-tumoral stroma contributes wild-type products to the tumour extracts. Excess stromal RNA would have increased towards unity the ratio of wild-type transcript to mutant, however, whereas the majority of the tumours studied here showed ratios substantially less than unity. Further, the ratio of wild-type to mutant transcripts in the xenografted tumours, in which the stromal component is negligible, covered the same range of values as in authentic primary tumours.

Many previous studies on a variety of animal and human tissues have indicated that mutational activation of *ras* genes is frequently an early event in the evolution of tumours (1,5-7,21). There is evidence that *ras* mutations may be influential at later stages of tumour progression also (22,23). In experiments in which mutant *ras* genes are selectively inactivated by specific recombination events, the pre-existing tumour phenotype reverts to a more normal form (23), indicating that mutated *ras* genes are necessary, in these cells at any rate, for the maintence of malignant behaviour. On the other hand, the mere possession of a mutated *ras* gene is not sufficient for malignant transformation of some cell types (5,6,12,25). Factors limiting its transforming potency appear to include its level of expression and that of any wild-type *ras* gene in the same cell. We interpret the data presented

here to imply that expression of the Ki-*ras* oncogene may confer some survivorship or growth advantage upon tumour cells even in well-established tumours. In contrast, expression of the wild-type transcript may be diminished, because it is unnecessary for, or limiting to growth. It will be of interest to determine whether these early results are repeated in larger series of cases, and in particular whether the ratio of wild-type to mutant transcript differs between carcinomas and adenomas.

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