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High frequency of K-*ras* mutations in sporadic colorectal adenomas

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Abstract

The frequency of activating mutations at codons 12 and 13 of the K-*ras* gene was investigated in 57 sporadic adenomas from 47 patients using the polymerase chain reaction and oligonucleotide hybridisation assay. Sixty eight per cent of the adenomas tested were positive for K-*ras* mutations. This high frequency, combined with the lack of a correlation between mutations and adenoma size, suggest that K-*ras* mutations occur earlier in the adenoma-carcinoma sequence than has previously been suggested. The high frequency observed in sporadic adenomas contrasts with the reported low frequency (18%) in adenomas from patients with familial adenomatous polyposis (FAP), suggesting a possible difference in the molecular genesis of FAP and non-FAP adenomas. Finally, it was found that adenomas from patients with a personal history of colorectal cancer were more likely to contain a K-*ras* mutation than those from patients with no such history. This is a new finding and worthy of further study.
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Colorectal neoplasia is an attractive model of multistep tumorigenesis in which abnormalities of a number of dominant oncogenes and tumour suppressor genes have been identified as frequent events.[1] One of the most striking findings is the high frequency of K-*ras* mutations in colorectal cancers—first reported by Bos *et al*[2] and Forrester *et al*[3], and confirmed in many subsequent studies (Table I). Most of the reports concern colorectal carcinomas and there is less information about the principal precursor lesion, the colorectal adenoma. The frequency of K-*ras* mutations reported in the various studies is fairly consistent for both sporadic colon cancers (377 of 940 (40%) and cancer associated with familial adenomatous polyposis (FAP) (20 of 54 (37%)), an autosomal dominant disease with a predisposition to colorectal cancer.

The reports on the frequency of *ras* mutations in FAP adenomas are in reasonable agreement with an overall frequency of 18% (53 of 296). There is, however, considerable variation in the reported frequency of *ras* mutations in non-FAP adenomas, with frequencies ranging from 15 to 75%. Some of this variation may be caused by the sensitivity of the detection method used as well as the particular types of *ras* mutation for which the assay was performed but it is also possible that differences in the patient populations analysed could contribute. Most of the studies have examined patients in North America and there is little information for European patients.[6] The frequencies of *ras* mutation in adenomas and carcinomas are important clues to the timing of mutations in colorectal carcinogenesis.

The data on FAP tumours suggest that *ras* mutation is not one of the very early steps in tumorigenesis, as the frequency of *ras* mutations in FAP adenomas is generally lower than that in FAP cancers (18% *v* 37%). Miyaki *et al*[8] showed that the frequency of K-*ras* mutation increased during the development from moderate to severe dysplasia and with the size of the adenomas in FAP.

With regard to non-FAP tumorigenesis, the situation is much less clear (Table I). Therefore, the objective of this study was to assess the role of *ras* mutations in non-FAP colon carcinogenesis by determining (a) the frequency of K-*ras* mutations in a large series of non-FAP adenomas in European patients and (b) whether or not there is any correlation between presence of K-*ras* mutations and one or more of the following variables: size of the adenoma, anatomical location, microscopic and macroscopic growth pattern, degree of dysplasia, personal history of colorectal cancer, and gender of the patient.

TABLE I *Summary of results of studies investigating the frequency of ras mutations in colorectal tumours*

Lesion	*Mutation**	*Method†*	*Incidence‡ % (no)*	*Reference no*
Adenomas	all ras	PCR-OH	50 (40)	1
Adenomas	K-12 & 13	PCR-seq	75 (12)	4
Adenomas	K-12	PCR-RE	15 (13)	5
Adenomas	K-12 & 13*	PCR-RE	28 (7)	6
FAP adenomas	all ras	PCR-OH	7 (75)	7
FAP adenomas	K-12	PCR-OH	22 (111)	8
FAP adenomas	K-12	PCR-OH	12 (51)	9
FAP adenomas	K-12	PCR-RE	30 (59)	5
Cancers	all but H-13	PCR-OH	41 (27)	2
Cancers	all ras	PCR-OH	47 (92)	1
Cancers	K-12 & 13	PCR-seq	65 (40)	4
Cancers	K-12 & 13	PCR-OH	40 (129)	10
Cancers	K-12 & 13*	PCR-RE	27 (15)	11
Cancers	K-12	Western	57 (7)	12
Cancers	K-12 & 13	PCR-seq	52 (21)	13
Cancers	K-12 & 61	PCR-OH	24 (58)	14
Cancers	all ras	PCR-OH	27 (30)	15
Cancers	K-12	PCR-RE	25 (68)	5
Cancers	K12 & 13*	PCR-RE	42 (33)	6
Cancers	K-12 & 13	PCR-OH	38 (86)	16
Cancers	K-12 & 13	PCR-OH	39 (99)	17
Cancers	all ras	PCR-OH	32 (50)	18
Cancers	K-12 & 13	PCR-seq	48 (24)	19
Cancers	K-12, 13 & 61	PCR-OH	37 (54)	20
Cancers	K-12 & 13	PCR-seq	42 (36)	21
Cancers	K-12	PCR-OH	54 (71)	22
FAP & non-FAP cancers	K-12	RNAse MM cleavage	40 (66)	3
FAP cancers	K-12	PCR-OH	44 (26)	8
FAP cancers	K-12	PCR-OH	35 (20)	9
FAP cancers	K-12	PCR-RE	38 (8)	5

*Types of ras mutations assayed for. All ras=K, H, and N-ras at codons 12, 13 and 61; **only codon 13 glycine to aspartic acid encoded mutations assayed for.
†Method used to detect ras mutations. PCR=polymerase chain reaction; OH=oligonucleotide hybridisation; RE=restriction enzyme; MM=mismatch cleavage; seq=sequencing.
‡percentage of adenomas with ras mutation; ( ) number of adenomas assayed.

TISSUE AND DNA PREPARATION

Fifty seven sporadic adenomas from 47 patients (32 men and 15 women) ranging in age from 33 to

93 years (mean (SEM)=67 (1·6)) were analysed. The paraffin embedded tissue blocks were selected from the archives of St Mark's Hospital, London. One to three 5 μm sections were cut from each block and placed in 1·5 ml Eppendorf tubes. DNA was extracted by the method of Jackson *et al.*[23]

DNA AMPLIFICATION

DNA samples were amplified by the polymerase chain reaction (PCR) techniques using a pair of 20-mer oligonucleotides which flank a region of exon 1 including the 12th and 13th codons of the human K-*ras* gene:

5′ CCTGCTGAAAATGACTGAAT 3′ and
5′ TGTTGGATCATATTCGTCCA 3′

100 μl of PCR mix (10 mM Tris (pH 8·3), 50 mM KCl, 2·5 mM $MgCl_2$, 1·5 mM of each dNTP, 1 μmol of each primer, and 2·5 units of Taq DNA polymerase (Boehringer)) were added to each DNA containing tube. After denaturation at 95°C for 5 minutes the samples were subjected to 40–50 cycles of amplification using a thermal cycler (Hybaid). Each cycle comprised 1 minute denaturation at 95°C, 1 minute annealing at 55°C, and 1 minute elongation at 72°C. The final cycle included an elongation step of 4 minutes at 72°C to ensure that all double stranded product was full length. The resulting amplified mixtures were run on 2·5% agarose gels to confirm the presence of the expected 115 bp amplified product.

MUTATION ANALYSIS

The amplified DNAs were divided into seven equal aliquots and slot-blotted onto nylon membranes (Hybond N, Amersham, UK) using a Bio-Dot SF Microfiltration Apparatus (BIO-RAD, UK). Seven replicate filters were prepared and the DNA was fixed by ultraviolet illumination. Probe preparation and hybridisation protocols were as described by Farr *et al.*[7] Briefly, six 20–mer probes homologous to the six possible point mutations that would substitute the 12th amino acid and one homologous to wild type codon 12 were end labelled with [gamma-^{32}P]ATP and T4-polynucleotide kinase. A complete list of the sequences of the probes is available upon request. Each probe was then hybridised to one of the seven replicate filters for 3 hours at 55°C. Afterwards the filters were washed three times in 2x SSPE/0·1% SDS for 10 minutes each at room temperature. They were then washed at 59°C in 3 M tetramethyl ammonium chloride (TeMAc) buffer for 30 minutes. Filters were exposed to Fuji RX film at −70°C using intensifying screens. Filters were stripped by washing them in boiling 0·1% SDS for 30 minutes. The procedure was repeated using a different probe for each filter, in order to confirm the results and then a similar procedure was used to probe the filters for point mutations at the first two positions of codon 13. Only mutations at codons 12 and 13 of the K-*ras* gene were assessed as these particular mutations have been consistently shown to comprise at least 90% of the *ras* mutations in colorectal tumours.

SEQUENCING OF AMPLIFIED PRODUCT

An additional 5 μm section from one of the paraffin blocks was cut and the DNA extracted and amplified as described above. 60 μl of amplified product were run on a 2·5% agarose (Nusieve) gel. The 115 bp band was cut out and the DNA was eluted from the gel using an Extraphor electrophoretic concentrator (Pharmacia, UK). Approximately 200 ng of purified DNA was ligated with ddT-tailed pBluescript II KS+ vector which was then used to transform XL1-Blue cells. A plasmic mini-prep was prepared for double-stranded sequencing of the insert. T3 DNA polymerase was used and the sequencing conditions were as recommended by the manufacturer (Sequenase version 2·0 kit, United States Biochemical).

ASSESSMENT OF CLINICOPATHOLOGICAL VARIABLES.

The variables assessed and correlated with *ras* mutation data were as follows:

(1) Size of the adenoma. This was assessed by measuring the diameter of the lesion after fixation. The sizes categories used were the same as those used in other studies to facilitate direct comparison of results (less than 1 cm diameter; 1–2 cm diameter; more than 2 cm diameter).

(2) Anatomical location: left or right sided.

(3) Degree of dysplasia (high, moderate, or low) was assessed independently by three pathologists.

(4) Micro-growth pattern (tubular, tubular-villous, or villous) was also assessed independently by three pathologists.

(5) Macro-growth pattern (sessile or pedunculated) was assessed by JPS.

(6) Personal history of colorectal cancer. If the patient had had a colorectal cancer before the removal of their adenoma assessed in this study or had a cancer at the time of removal of their adenoma then they were classified as being positive for the presence of cancer. No personal history of colorectal cancer defined those classified as negative for presence of cancer.

(7) Gender of the patient.

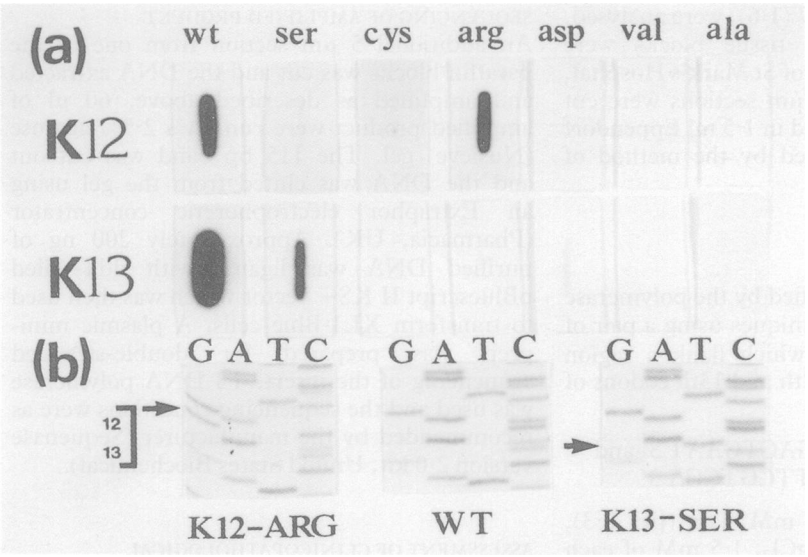
STATISTICAL ANALYSIS

Associations between variables were tested using the χ^2 test. The χ^2 test for trend was performed when a linear relationship was expected. The relationship between presence of *ras* mutation and all other variables was investigated using logistic regression analysis. The final model was determined by forward stepwise variable selection procedure. Only variables found to be significant at the 5% level were entered into the model at each step.

Results

By slot-blot analysis, 68% (39) of the adenomas tested were positive for a mutation, with three of these positive for a mutation at both codons 12 and 13. To verify the slot-blot result of these apparent double mutants one of them was confirmed by sequencing (Figure).

Table II presents the results of tests for



*Slot-blot and sequence analysis of polymerase chain reaction amplified DNA extracted from a single colorectal adenoma for activating mutations of codons 12 and 13 of the human K-*ras *gene. (a) The autoradiographs of the filters show that the filters hybridised with codons 12 and 13 wild type specific probes (GGT-GLY and GGC-GLY, respectively). In addition, two of the mutant specific probes gave detectable signals: CGT-ARG at codon 12 and AGC-SER at codon 13. (b) Sequencing autoradiograms of the antisense strands of three clones show the existence of the three alleles inferred from (a) above: mutant at codon 12 (ACG'-ARG); wild type at codons 12 (ACC'-GLY) and 13 (GCC'-GLY); and mutant at codon 13 (GCT'-SER). Arrows denote mutant bases.*

association between the presence of *ras* mutation and all other variables. Although there seemed to be a high frequency of mutations (Table III) in small (<1 cm) and in large (>2 cm) adenomas, and a lower frequency in the intermediate group (1–2 cm), this was not statistically significant (p=0·779) (see Table II). Only an association with personal history of colorectal cancer was found to be statistically significant (p=0·044) (see also Table IV). We also obtained similar results using logistic regression analysis. Therefore, the final model contains only one variable: personal history of colorectal cancer (odds ratio=3·365; 95% confidence interval=1·003, 11·285).

TABLE II *Results of tests for an association between the presence of ras mutation and all variables assessed*

*Variable**	*p value*
Personal history of cancer	0·044
Degree of dysplasia†	0·453
Size of adenoma†	0·779
Micro growth pattern	0·327
Macro growth pattern	0·845
Anatomical location	0·153
Gender of patient	0·394

*Refer to 'methods' for a definition of each variable.
†χ^2 test for trend.

TABLE IV *Relation between ras mutations and personal history of colorectal cancer*

Personal history	*Ras mutation*	*No ras mutation*	*Total**
Yes	22	5	27
No	17	13	30
Total	39	18	57

*Number of adenomas analysed.

TABLE V *Frequency of the various types of K-*ras *mutations*

Mutation	*Total* no (%)*
K-12 serine	0 (0)
K-12 arginine	14 (33)
K-12 cysteine	0 (0)
K-12 asparagine	3 (7)
K-12 alanine	0 (0)
K-12 valine	17 (40)
K-13 serine	5 (12)
K-13 arginine	0 (0)
K-13 cysteine	1 (2)
K-13 asparagine	1 (5)
K-13 alanine	0 (0)
K-13 valine	0 (0)
Totals	42

*Number of adenomas positive for each type of mutation; (%)= number of adenomas positive for each type of mutation as a percentage of the total number of K-*ras* positive adenomas.

Most of the total of 42 mutations detected occurred at codon 12 rather than at codon 13 (that is, 81% *v* 19%). The occurrence of each of the 12 types of *ras* mutation for which an assay was performed is summarised in Table V. The two most common mutations occurred at codon 12 and resulted in replacement of glycine (codon GGT) with valine (codon GTT) or arginine (codon CGT) (40% or 33% of all mutations, respectively). The frequency of mutations at codon 12 resulting in replacement of glycine by aspartic acid was 7%, which is much lower than expected given the findings of previous reports.

Discussion

This study had two aims: firstly, to determine the frequency of activating mutations at codons 12 and 13 of the K-*ras* gene in a large UK series of non-FAP adenomas; and secondly, to determine if the presence of a *ras* mutation is correlated with any clinical or pathological variables that could shed light on the role of these mutations in colorectal carcinogenesis. It was found that 68% of the adenomas contained a mutation at either codon 12 or 13 of the K-*ras* gene. This frequency is in general agreement with most studies from the USA (for instance, Burmer and Loeb[4] reported a 75% incidence and Vogelstein *et al*[1] a 50% incidence) and consider-

TABLE III *Relation between mutations in codons 12 or 13 of the K-*ras *gene and size of the adenoma*

		*Positive**			*Negative†*			
Size	*Dysplasia*	*Tubular*	*Tub/vil*	*Villous*	*Tubular*	*Tub/vil*	*Villous*	*Total*
<1 cm	Low	12	2	1	3	0	1	19
	Low/high	1	0	0	0	1	0	2
	High	1	0	1	0	0	0	2
1–2 cm	Low	6	2	1	4	2	2	17
	Low/high	0	1	1	0	0	0	2
	High	0	0	0	1	1	0	2
>1 cm	Low	4	0	3	0	1	1	9
	Low/high	0	0	3	0	0	0	3
	High	0	0	0	0	0	1	1
Total		24	5	10	8	5	5	57

*Number of adenomas positive for a K-*ras* mutation by oligonucleotide hybridisation assay.
†Number of adenomas negative for a K-*ras* mutation by oligonucleotide hybridisation assay.

ably higher than the incidences reported from Japan (Ando *et al*[5] reported 15%) and the UK (Bell *et al*[5] reported 28%). The differences in frequency may reflect ethnic variation or differences in dietary habits in various parts of the world, but most of the studies are relatively small and more data are needed before we can draw inferences with confidence.

The finding that most mutations occurred at codon 12 rather than codon 13 (81% *v* 19% of all detected mutations) agrees with the findings of others. There was, however, a difference in the type of mutation detected. In the present study, mutations resulting in the replacement of glycine by valine or arginine were the most frequent (40% and 33% respectively). In contrast, American studies have generally found that mutations encoding aspartic acid, valine, and serine are the most common. This difference in the type of mutations detected may reflect the type of carcinogen exposure in different areas of the world.

The finding of three adenomas that contained both a codon 12 and a codon 13 mutation in their K-*ras* genes is not a new one. Forrester *et al*[24] reported a villous adenoma which contained a K-*ras* and an N-*ras* activating mutation. Furthermore, Farr *et al*[7] have found two FAP adenomas which each contained two codon 12 mutations in the K-*ras* gene; one resulting in the replacement of glycine by valine and the other in the replacement of glycine by aspartic acid. In the present study, one of the double mutants detected by oligonucleotide hybridisation was sequenced and it was found that the mutations occurred on separate alleles. What these double mutants mean in terms of the pathogenesis of colorectal adenomas is not known, but we suggest three possibilities. Firstly, *ras* was not the initiating mutation in these adenomas but occurred subsequently and in two distinct cells within the growing population of initiated cells. Secondly, the mutations, although on separate alleles, are both in the same cell, that is one *ras* mutation occurred early and conferred a growth advantage and then the remaining wild type allele was subsequently also mutated. Thirdly, the adenoma arose from two distinct cell populations.

Two interesting observations were made when analyses were done to determine if *ras* mutations were correlated with one or more of the following variables: adenoma size, anatomical location, microscopic and macroscopic growth pattern, degree of dysplasia, personal history of colorectal cancer, and gender of the patient. Firstly, *ras* mutations were significantly correlated with a personal history of colorectal cancer. In other words, if a colorectal cancer was present at the time the adenoma was removed or had been diagnosed before removal of the adenoma, the adenoma was more likely to contain a *ras* mutation than if the patient had no history or evidence of colorectal cancer at the time of adenoma removal. To the best of our knowledge, a correlation of *ras* mutations in non-FAP adenomas with a personal history of colorectal cancer is a new finding, possibly because other studies have not assessed this variable for an association.

What does the correlation between *ras* mutation and a personal history of colorectal cancer suggest about the role of *ras* mutations in non-FAP colorectal carcinogenesis? Epidemiological evidence indicates that the environmental factors responsible for the formation of adenomas differ from those that cause adenomas to progress to malignancy.[25] It is therefore possible that adenomas which progress to cancer do so because they have been exposed to additional mutagens compared with those adenomas which do not. It would follow from this theory that synchronous or subsequent adenomas from colorectal cancer patients would also have been in the epithelial field exposed to the mutagens and would subsequently be more likely to have additional mutations in their adenomas, such as K-*ras* mutations. Such a scenario is supported by a recent study[26] which found frequent K-*ras* mutations in biopsy specimens from areas of high grade dysplastic epithelium from patients with ulcerative colitis, particularly when a cancer was present elsewhere in the large bowel.

The other interesting finding was that *ras* mutations were not correlated with the size of the adenoma. This is in contrast to the report of Vogelstein *et al*[1] who found that adenomas greater than 2 cm in diameter had a higher frequency of *ras* mutations than those less than 2 cm. Possibly *ras* mutations are an earlier event in sporadic adenoma formation than was previously thought.

In conclusion, the high frequency of K-*ras* mutations in non-FAP adenomas (68%) of this UK patient population, combined with the lack of correlation between *ras* mutations and adenoma size, suggest that *ras* mutations occur earlier in the cancer process than has previously been suggested.[1] Furthermore, the higher frequency of K-*ras* mutations in non-FAP adenomas compared with the overall reported frequency in FAP adenomas (18%) suggests that there are differences in the molecular pathogenesis of these two types of adenomas, just as it has been reported that there are differences in the molecular pathogenesis of sporadic colorectal carcinomas and ulcerative colitis associated carcinomas.[6] Finally, the finding that adenomas from patients with a personal history of colorectal cancer are more likely to contain a K-*ras* mutation than those with no such history is a new one and worthy of further study.

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